THE EFFECT OF STROPHANTHIN-G ON THE RATE OF DEAMINATION OF ADENYL COMPOUNDS

by W. G. NAYLER[1]

(From the Baker Medical Research Institute, Melbourne).

(*Accepted for publication 2nd December, 1958.*)

SUMMARY.

The action of strophanthin-G on the deaminase systems present in freshly shed blood and extracted from mouse cardiac muscle has been studied.

No change in adenosine or adenylic acid deaminase activity was noted following the addition of the glycoside.

Adenosine depressed the isolated toad heart. This depressant effect was not significantly modified by a sub-threshold level of strophanthin-G.

These results are discussed in relation to the possible involvement of deaminase activity in the inotropic response produced by the glycosides.

INTRODUCTION.

In 1955 Rand, Stafford and Thorp noted that a sub-threshold dose of ouabain increased the E.C.G. irregularities recorded following the injection of adenosine as well as adenyl compounds into the atrium of anaesthetized guinea pigs and suggested that this effect was part of the mechanism involved in the positive inotropic activity of the cardiac glycosides. They postulated that the glycosides inhibit the enzymes responsible for deamination of adenosine and adenosine-containing compounds so that the inotropic activity of the glycosides is due to the accumulation of small concentrations of adenyl derivatives.

If this hypothesis is valid then the activity of the deaminase systems present in isolated cardiac muscle should be inhibited or depressed by the glycosides. Also adenosine base as well as adenosine polyphosphate compounds should exert a positive inotropic effect on the isolated heart and this response should be increased by the glycosides.

Snellman and Gelotte (1950) reported that 'Digitotal' inhibited the deamination of a substance having the characteristic absorption spectrum of adenosine. They located the enzyme activity in cardiac actin and believed their adenosine-containing compound to be adenosine triphosphate. Earlier, Conway and Cooke

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

(1939a) failed to demonstrate any adenosine triphosphate deaminase activity in either atrial or ventricular tissues.

A transient positive inotropic response associated with bradycardia and increased coronary arterial dilation following a small dose of adenosine has been reported by some workers (Drury, 1936). Others have recorded depressant effects following the administration of both adenosine and some adenyl-containing compounds (Lutwak-Mann, 1939; Gillespie, 1934).

The present study was undertaken to determine whether or not strophanthin-G inhibited or depressed the enzyme systems responsible for the deamination of adenosine and adenyl compounds both in mouse isolated cardiac muscle and in freshly shed human blood (Conway and Cooke, 1939b). In addition, some observations were made on the effect of adenosine on the isolated toad heart.

Method.

Throughout these experiments the rate of ammonia liberation was used as an index of deaminase activity both in muscle extracts and in freshly shed blood. Ammonia determinations made by the modified Conway technique described by Brown *et al.* (1957) were standardised against dilutions of a stock solution of ammonium sulphate containing 0·2090 mg.N/ml. as estimated by the standard Kjeldahl analysis. Optical densities were read in a Hilger Uvispec Spectrophotometer and a standard curve constructed from 77 determinations. Estimations were made in duplicate and incubations carried out at 37·0° C. Where enzyme extracts were prepared from isolated myocardial tissue mice were killed by a blow on the head and the hearts rapidly removed.

Adenosine deaminase.

Adenosine deaminase extracts were prepared from mouse hearts by the method of Conway and Cooke (1939a) and their activity tested at 37.0° C. using 6 mgm. adenosine base substrate[2] per 4 ml. of aqueous enzyme extract. In twelve preparations duplicate determinations of the ammonia liberated after ten minutes' incubation were made and compared with those from a similar series in which strophanthin-G[3] was initially added to the enzyme extract to give a final concentration of 10 μg./ml.

In a second series adenosine deaminase was extracted and tested following the method of Brady (1942). Adenosine and strophanthin-G concentrations were as above and duplicate ammonia determinations made after ten and twenty minutes' incubation at 37·0° C. both in the presence and absence of the glycoside — in each case six experiments were made.

Adenylic acid deaminase.

The activity of adenylic acid deaminase extracted from freshly excised mouse hearts by the method of Lu and Needham (1941) was tested, using M/20 citrate buffer and 6 mg. adenylic acid[4] substrate per 4 ml. of enzyme extract. The effect of strophanthin-G (10 μg./ml.) on the deaminase activity after ten and twenty minutes' incubation was determined in fourteen and nine experiments respectively.

---

[2] Adenosine: BDH product.
[3] Strophanthin-G: As Ouabaine Arnaud. Laboratoire Nativelle Product.
[4] Adenosine monophosphate: Sigma product.

Blood ammonia.

Conway and Cooke (1939b) demonstrated that the initial increase in ammonia concentration of freshly shed blood was due to adenosine or adenylic acid deaminase activity. Hence the action of strophanthin-G (1 μg./ml.) on these deaminase systems was studied.

Strophanthin-G (1 μg./ml.) was added to blood immediately after its withdrawal from an arm vein of a resting human subject and ammonia determinations made after two, five and ten minutes had elapsed. Care was taken to avoid haemolysis and only free-flowing blood was sampled. In some experiments blood was taken directly into a syringe containing the required amount of the glycoside and in others the blood was quickly transferred to a tube containing strophanthin-G. All determinations were made in duplicate and the time measured from the moment blood entered the syringe. The ammonia concentrations found in the glycoside-enriched blood were compared with those found in untreated blood after similar time intervals.

Action of adenosine on the isolated heart.

Adenosine base was added to a series of "summer" and "winter" recycling toad hearts (*Bufo marinus*) and a kymograph record of the force of ventricular contraction used as an index of response. The isolated hearts, perfused with toad Ringer (Nayler and McKelvie, 1956) were used immediately after dissection. Adenosine dissolved in Ringer solution was added to give a final concentration of 1 μg./ml. in six "summer" preparations and its effect on the force of contraction recorded throughout the following fifteen minutes. In a further similar series the adenosine concentration was raised to 10 and 100 μg./ml. and the effect recorded as above.

The effect of sub-threshold doses of strophanthin-G on the response of isolated "summer" and "winter" hearts to adenosine was investigated in another series of twelve hearts. Thus in six "summer" hearts the effect of strophanthin-G (1 μg./ml.) on the response to 10 and 100 μg./ml. of adenosine was recorded. In a similar series of experiments using "winter" hearts the strophanthin-G level was raised to 10 μg./ml. — a sub-threshold dose level (Nayler, 1957), and the response to 1 and 100 μg./ml. of adenosine again recorded.

RESULTS.

The method described by Brown *et al.* (1957) for micro estimations of ammonia proved reliable throughout the range encountered in these experiments. Care was taken to avoid any ammonia contamination and it was necessary to use "all-glass" distilled water throughout.

Adenosine deaminase.

Strophanthin-G (10 μg./ml.) failed to produce any significant alteration in the rate of adenosine deaminase activity. In Fig. 1 the results obtained from those experiments in which the enzyme was extracted according to the method of Conway and Cooke are summarized. Whereas adenosine deaminase liberated 0·38 mg. of ammonium nitrogen/gm. wet weight after ten minutes' incubation with 6 mg. of adenosine base, 0·37 mg. were liberated in the presence of strophanthin-G. In those experiments in which Brady's extraction method was used a similar result was obtained – see Fig. 2. Thus after ten minutes' incubation with adenosine alone 0·09 mg. ammonium nitrogen/gm. wet weight was liberated compared with 0·14 mg. in the presence of the glycoside.

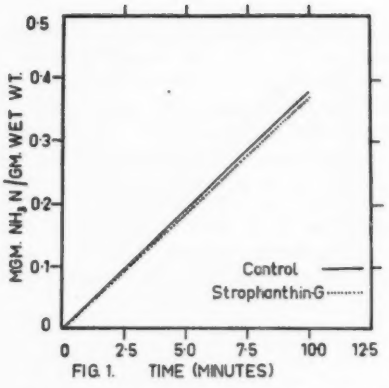


FIG. 1.

Fig. 1. Effect of strophanthin-G (10 µg./ml.) on ammonia liberation from adenosine following ten minutes' incubation with adenosine deaminase extracted from mouse myocardium (Conway and Cooke, 1939a). In both cases the mean results of twelve experiments are displayed.

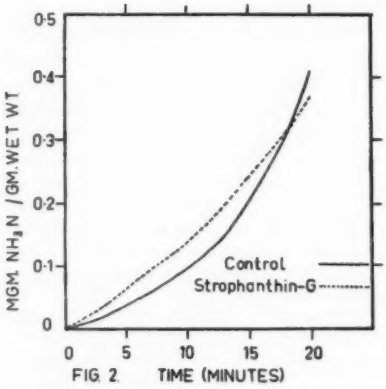


FIG. 2.

Fig. 2. Effect of strophanthin-G (10 µg./ml.) on ammonia liberation from adenosine following ten and twenty minutes' incubation with adenosine deaminase extracted from mouse myocardium (Brady, 1942). In each case the mean results of six experiments are displayed.

Adenylic acid deaminase.

The effect of strophanthin-G on the activity of adenylic acid deaminase is summarised in Fig. 3. Fourteen determinations were made after ten minutes' incubation and nine after twenty minutes both in the presence and absence of the glycoside. At both times the rate of ammonia liberation from the glycoside-enriched preparation slightly exceeded that of the deaminase-adenylic acid mixture.

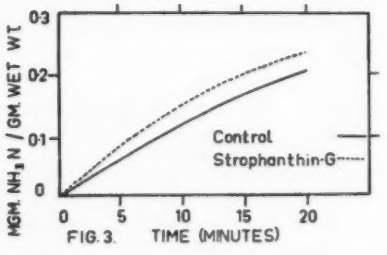


FIG. 3.

Fig. 3. Effect of strophathin-G (10 µg./ml.) on the activity of adenylic acid deaminase extracted from mouse myocardium (Lu and Needham, 1941). Six determinations were made after ten and nine after twenty minutes' incubation.

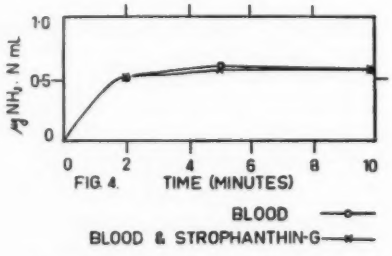


FIG. 4.

Fig. 4. Effect of strophanthin-G (1 µg./ml.) on ammonia liberated from human blood two (16 determinations), five (8 determinations) and ten minutes (8 determinations) after shedding.

Blood ammonia.

The rate of ammonia formation in freshly shed blood was not affected by the presence of strophanthin-G. The mean results obtained in a series of experiments are displayed in Fig. 4, determinations having been made two minutes, five minutes and ten minutes after withdrawal of blood from the vein.

Adenosine and the isolated heart.

Adenosine 1-100 μg./ml. depressed both "summer" and "winter" isolated hearts – see Fig. 5, where a typical record of the ventricular force of contraction is shown. Adenosine in the above concentration produced depression prior to the onset of bradycardia and cardiac irregularities, and this depression was apparently not modified by a subactive dose of strophanthin-G – see Fig. 6. Smaller doses of adenosine – 1-10 μg./ml.–consistently failed to produce a positive inotropic effect on the isolated heart, even in the presence of sub-threshold levels of the glycoside.

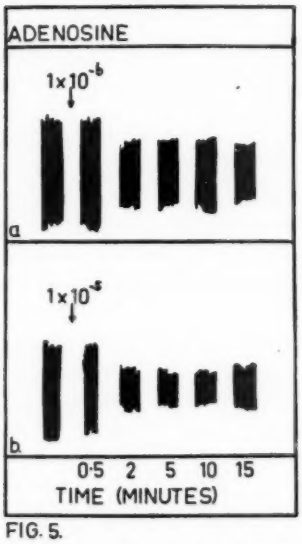


FIG. 5.

Fig. 5. Effect of adenosine (1-10 μg./ml.) on the recycling "summer" toad heart. Kymograph record.

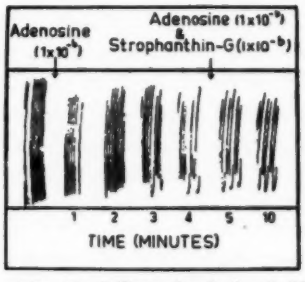


Fig. 6. Effect of sub-threshold dose of strophanthin-G (1 μg./ml.) on the depressant action of adenosine (1 μg./ml.) on the recycling "winter" toad heart. Kymograph record.

DISCUSSION

The results obtained in these experiments indicate that strophanthin-G has little, if any, effect upon the deaminase enzymes extracted from mouse myocardium or those present in freshly shed blood. They therefore provide no evi-

dence to support the hypothesis proposed by Rand *et al.* that the positive inotropic effect of the glycoside is due to the accumulation of small concentrations of adenosine or adenyl derivatives. Further a depressant action of adenosine on the isolated toad heart was consistently recorded in this series and subthreshold doses of strophanthin-G failed to produce any marked change in the adenosine-induced depression.

Conway and Cooke (1939), Ostern and Mann (1933) and others have demonstrated the presence of enzymes capable of deaminating adenosine and muscle adenylic acid in both skeletal and cardiac muscle. Conway and Cooke failed to demonstrate any deamination of the higher adenyl phosphate compounds in skeletal or cardiac muscle but more recently Snellman and Gelotte (1950) reported a deaminase associated with cardiac actin which acted on an adenyl-containing compound they believed to be adenosine triphosphate. Crude aqueous extracts of mouse myocardium show slight adenosine triphosphate deaminase activity which is not changed by presence of strophanthin-G (Nayler, unpublished results).

Lu and Needham (1941) demonstrated that the adenylic acid deaminase of skeletal muscle was 500-1000 times more active than that of cardiac muscle or other tissues. This observation has recently been confirmed by Ito and Grisolia (1957) whose acetone extracts of adenylic acid deaminase from cardiac muscle showed only 1/1000 the activity of that extracted from skeletal muscle. Since the glycosides fail to produce any effect upon skeletal muscle it seems unlikely that adenylic acid deaminase of cardiac muscle is involved in the inotropic activity of the glycosides. The results of this present series similarly indicate that the more active adenosine deaminase of cardiac muscle is not involved in the inotropic activity of strophanthin-G.

In 1952 Greiner reported that the improved contractile force following digitilization of the cat papillary muscle was associated with the restoration of adenosine triphosphate content to normal levels. Earlier Weicker (1935) noted a raised level of both phosphocreatine and adenosine triphosphate in cat hearts following treatment with digitalis. On the other hand, other workers have failed to find any change in adenosine polyphosphate levels following glycoside therapy. Wollenberger (1951) using the dog heart-lung preparation found no change in adenosine triphosphate or phosphocreatine levels and Rebar *et al.* (1957) reported an actual decrease in phosphocreatine along with an unchanged adenosine polyphosphate concentration in dog ventricle.

If there is in fact an increase in level of adenosine-containing compounds associated with glycoside treatment, then according to the above results some mechanism other than a changed rate of destruction *via* the deaminase systems must be sought.

Hollander and Webb (1957) when studying the effect of adenosine nucleo-

tides on the contractility and membrane potentials of the rat atrium observed that both nucleotides and nucleosides depressed the rat atrium contractility. They noted that adenosine tri-, d- and mono-phosphates and adenosine base acted as atrial depressants of equal potency. This adenosine-induced depression has been reported by other workers (Dubois-Ferriere, 1951; Gillespie, 1934; Chevillard and Guerin, 1955) and is confirmed in the above results. Some earlier reports suggest slight "beneficial" activation on the isolated hearts, but this occasional stimulant effect was probably due to coronary vasodilation or the presence of impurities–Drury (1936). Since sub-threshold doses of strophanthin-G failed to modify the depressant action of adenosine in the present series and since adenosine base produced only a negative inotropic effect it is difficult to explain the positive inotropic activity of the glycosides in terms of the accumulation of adenosine or adenosine-containing compounds.

Associated with the adenosine depression of the myocardium Hollander and Webb (1957) noted an increased membrane permeability to potassium ions. They found that the purine-ribose moiety was the basic unit required for activity and that combination of this group with unidentified sites in the membrane resulted in an increased permeability to potassium. The exaggerated E.C.G. irregularities following glycoside treatment reported by Rand *et al.* can be explained in terms of this altered permeability. In earlier experiments it was shown by varying the potassium concentration of the perfusate and by using quinidine sulphate to alter membrane permeability that the rate of onset and magnitude of the inotropic response due to the glycosides was related to the permeability of the membrane to potassium ions (Nayler, 1958).

Acknowledgment. I wish to thank Dr. T. E. Lowe for his continued encouragement with this project.

REFERENCES.

Brady, T. (1942): Biochem. J., 36, p. 478.
Brown, R. H., Duda, G. D., Korkes, S., and Hander, P. (1957): Arch. Biochem. Biophys., 66, p. 301.
Chevillard, L., and Guerin, J. (1955): C.R. Soc. biol., 149, p. 235.
Conway, E. J., and Cooke, R. (1939a): Biochem. J., 33, 1, p. 457.
Conway, E. J., and Cooke, R. (1939b): *Ibid.*, 33, 1, p. 479.
Drury, A. N. (1936): Physiol. Rev., 16, p. 292.
Dubois-Ferriere, H. (1951): Helvet. med. Acta., 18, p. 84.
Gillespie, J. H. (1934): J. Physiol., 80, p. 345.
Greiner, T. (1952): J. Pharmacol. exp. Therap., 105, p. 178.
Hollander, P. B., and Webb, J. L. (1957): Circ. Res., 5, p. 349.
Ito, N., and Grisolia, S. (1957): Experientia, 13, p. 442.
Lu, G. D., and Needham, D. M. (1941): Biochem. J., 35, p. 392.
Lutwak-Mann, C. (1939): Biol. Rev., 14, p. 399.
Nayler, W. G., and McKelvie, D. (1956): Austral. J. exp. Biol., 34, p. 239.
Nayler, W. G. (1957): *Ibid.*, 35, p. 131.
Nayler, W. G. (1958): *Ibid.*, 36, p. 323.

Ostern, P., and Mann, T. (1933): Biochem. Z., 260, p. 326.
Rand, M., Stafford, A., and Thorp, R. H. (1955): J. Pharmacol. exp. Therap., 114, p. 119.
Rebar, J., Rebar, B. T., and Ornachi, A. (1957): Circ. Res., 5, p. 504.
Snellman, O., and Gelotte, B. (1950): Nature, 165, p. 604.
Weicker, B. (1935): Arch. exper. Path. Parmakol., 178, p. 524.
Wollenberger, A. (1951): J. Pharmacol. exp. Therap., 103., p. 123.

A VIRUS OF THE ENCEPHALOMYOCARDITIS GROUP FROM A WATER-RAT, *HYDROMYS CHRYSOGASTER*, IN NORTH QUEENSLAND

by J. H. POPE

(From the Queensland Institute of Medical Research, Brisbane).

(*Accepted for publication 31st October, 1958.*)

SUMMARY.

A virus, referred to as the Innisfail strain, was isolated by mouse inoculation from a naturally infected water-rat, *Hydromys chrysogaster*, captured in north Queensland, Australia. It was shown to be indistinguishable from the EMC virus of Helwig and Schmidt by cross-neutralization and cross-complement fixation tests, and was considered to belong to the EMC group of viruses.

Mice and guinea-pigs were highly susceptible. Rats showed signs of paralysis, but usually survived. Rabbits and three-day-old chickens were resistant.

Infection of mice was characterised by paralysis and death in 2-3 days, and encephalitis and myositis were the prominent histopathological features. Heavily infected guinea-pigs died within 3-7 days, with encephalitis and severe macroscopic myocarditis.

This is believed to be the first report of natural infection with a virus of the EMC group in Australia.

Toxoplasma was also isolated from the water-rat, making a new host record.

INTRODUCTION.

The viruses commonly referred to as members of the encephalomyocarditis (EMC) group are immunologically indistinguishable (Warren, Smadel and Russ, 1949), and include Columbia-SK (Jungeblut and Sanders, 1940), MM (Jungeblut and Dalldorf, 1943), encephalomyocarditis (Helwig and Schmidt, 1945), and Mengo encephalomyelitis (Dick *et al.*, 1948) viruses. An informative account of the EMC group is given by Warren (1952).

At least some members of the EMC group are pathogenic for man. A human infection with the Mengo virus was diagnosed by isolation of the virus from the patient's blood (Dick *et al.*, 1948). Viruses considered to belong to the EMC group have been also isolated in Europe (Bieling, 1952; Verlinde and van Tongeren, 1953; Verlinde and Molron, 1954). Serological evidence of infection in man has been obtained in the Philippine Islands (Smadel and Warren, 1947), in Uganda (Dick *et al.*, 1948), in Surinam (Tiggelman-van Krugten and Collier, 1955), and in Europe (Hofman and Jungeblut, 1952; Keller and Vivell, 1952).

Hosts infected naturally or experimentally with EMC group viruses are listed in Table 1.

TABLE 1.

Known host range of EMC group viruses.

Natural hosts	Locality	Author
Cotton rat, *Sigmodon hispidus littoralis*	U.S.A.	Jungeblut and Sanders (1940)
Brown rat, *Rattus norvegicus**	U.S.A. & Canada	Warren, Russ and Jeffries (1949)
*R. rattus alexandrinus**		
Multi-mammate rat, *R. coucha**	Uganda	Horgan (1951)
Black rat, *R. rattus kijabius**		
Hamster	U.S.A.	Jungeblut and Dalldorf (1943)
Squirrel	U.S.A.	Kissling, Vanella and Schaeffer (1956)
Dog, *Canis familiaris**	Surinam	Tiggelman-van Krugten and Collier (1955)
Pig, *Sus scrofa**		
Cow, *Bos taurus**		
Mongoose, *Ichneumia* sp.	Uganda	Dick, Best, Haddow and Smithburn (1948)
Rhesus monkey, *Macacus rhesus*		
Chimpanzee, *Pan* sp.	U.S.A.	Helwig and Schmidt (1945)
Squirrel monkey, *Saimiri sciureus*	Colombia	Roca-Garcia and Sanmartin-Barberi (1957)
Night monkey, *Aotus trivirgatus*		
Mandrill baboon	U.S.A.	Kissling, Vanella and Schaeffer (1956)
Man, *Homo sapiens*	Uganda	Dick, Best, Haddow and Smithburn (1948)
Mosquitoes, *Taeniorhynchus fuscopennatus*		
Experimental hosts		
House mouse, *Mus musculus*		Jungeblut and Sanders (1940)
Field rats, *Lophuromys aquilis laticeps*		Kilham, Mason and Davies (1956a)
Tatera liodon smithi		
Arvicanthis abyssinicus nubilans		
Guinea-pig, *Cavia porcellus*		Dick (1948)
Chick embryo, *Gallus domesticus*		Helwig and Schmidt (1945)
Mongoose, *Mungos mungo macrurus*		Kilham, Mason and Davies (1956b)
African grey monkey, *Cercopithecus aethiops centralis*		Dick (1948)
Rabbit, *Oryctolagus cuniculus*		Warren (1952)

* Indicates diagnosis made by serological tests. In all other cases the virus was isolated.

A virus was isolated in mice from a water-rat, *Hydromys chrysogaster* Geoff., during a survey for *Toxoplasma* infections in the Innisfail district, north Queensland. It was designated as the Innisfail strain after the locality in which the rat was captured, and the results of a study that was made of it are reported here.

Methods and Materials.

The strain of mice used in this work was that previously described (Derrick *et al.*, 1954). Weaned mice were three weeks old at the time of inoculation, and infant mice less than three days old. Autopsies were performed aseptically. Selected tissues were triturated with sterile alundum, and suspended in physiological saline. For intraperitoneal inoculation of weaned mice 0·5 ml. of supernate was used, and for intracerebral inoculation 0·03 ml.

The EMC virus of Helwig and Schmidt[1] was in the form of 82nd passage mouse brain, and was subsequently maintained at —20° C., with occasional passages in mice.

Antisera to the Innisfail and EMC strains were prepared in laboratory rats, *Rattus norvegicus*, by one intraperitoneal inoculation of 0·1 ml. of infected mouse brain suspension (approximately 20 p.c.). With this dose, it was found that some rats showed signs of mild paralysis. Sera were collected 1-3 months after inoculation.

Neutralization tests were performed by the method of addition of dilutions of virus to constant volumes of serum. The mixtures of virus and serum were incubated in a water bath at 37° C. for one hour, then held in an ice-bath until inoculation. Six mice were each inoculated intracerebrally with 0·03 ml. of each serum-virus mixture, and were observed for 14 days. The LD_{50} was calculated according to the method of Reed and Muench (1938).

The method of preparing complement fixing antigen employed by Warren, Smadel and Russ (1949) proved suitable for use with the Innisfail strain, but not with the EMC strain. Consequently antigens for both strains were prepared from the brains of suckling mice, rather than of weaned mice, and proved quite effective. The titre of these antigens was of the order of 1/4.

The complement fixation test was carried out by the method of Lennette *et al.* (1949). Immune rat sera possessed complement fixing antibody in titres up to 1/64.

No evidence has been found of the presence of EMC virus in the mouse colony at this Institute, although a large number of routine mouse passages have been made by various routes of inoculation during the course of other work.

EXPERIMENTAL.

Pathogenicity of the Innisfail strain for laboratory animals.

Mice. On September 10, 1957, an autopsy was carried out on Rat 445, an apparently healthy female *Hydromys chrysogaster* captured on the bank of the North Johnstone River at Daradgee, near Innisfail. Two weaned mice were inoculated intraperitoneally with a suspension prepared from a pool of liver, spleen, kidney, lung, heart and brain of the rat. Eleven days later, both mice were hunched and showed flaccid paralysis of the hind legs which dragged limply beside the tail. They were otherwise quite alert, and the front quarters were unaffected. At fourteen days, one of the mice was still in the same condition. It was sacrificed, and successful serial passages were made. The other mouse recovered from the paralysis, and was sacrificed at 17 days, but subinoculated mice did not become affected.

In the second passage, a pool of liver, spleen, kidney, lung, heart and brain of the first passage mouse was inoculated intracerebrally into six mice and intraperitoneally into a second group of six. In the intracerebral group, all mice became paralysed, and either died or were sacrificed when moribund. On the other hand, only three mice became paralysed in the intraperitoneal group, and all survived. A further six mice were inoculated intraperitoneally with brain of the first passage mouse, and five became paralysed at 4-5 days. One of the

[1] Obtained through the courtesy of Dr. J. Orde Poynton, Director of the Institute of Medical and Veterinary Science, Adelaide.

paralysed mice was sacrificed for passage, and the other four died; the sixth mouse remained unaffected.

Another group of mice was inoculated subcutaneously with the pool of mouse tissues. The mice remained well, and were examined 36 days later. Splenomegaly and pneumonitis, typical of infection with *Toxoplasma gondii*, were present, and *Toxoplasma* was demonstrated in brain smears stained with Giemsa. Thus, Rat 445 was infected with *Toxoplasma* as well as with the Innisfail virus, this being a new host record for both. The possibility that the deaths in the mice were due to *Toxoplasma* was excluded, by showing that the infectivity remained after repeated freezing and thawing, a treatment that is lethal to toxoplasms.

The virus was subsequently maintained in mice by serial inoculation of brain by the intraperitoneal route, and the mortality was 100 per cent. In the tenth passage, the survival time was four days, but by the 20th passage it was reduced to 2-3 days. Paralysis was less evident with this brief survival time, but a crusty conjunctival secretion was regularly noted. Seventy-three intraperitoneal passages have so far been made in mice.

Guinea-pigs. The pool of tissues of the first passage mouse was inoculated intraperitoneally into six guinea-pigs, which were observed for 28 days. Two showed no abnormalities, two were febrile on the sixth day only, and another was febrile on the tenth day only, but they remained otherwise well. The sixth guinea-pig, which had been afebrile, and appeared quite well on the morning of the seventh day, was unexpectedly found dead a few hours later. Gross necrosis of the myocardium was seen at autopsy.

A high mortality followed inoculation of guinea-pigs with material from later mouse passages. Intraperitoneal inoculation of three guinea-pigs with brain of an infected mouse of the eighth passage resulted in death of all animals in 3-5 days. Similar inoculation of two guinea-pigs with mouse brain of the tenth passage was fatal for both in two days. No fever was recorded in four of the five guinea-pigs, while the fifth, which survived five days, had only one febrile day. Gross myocardial necrosis was observed in the two which died at two days; the other three were not autopsied.

Rats. The pool of tissues from the first passage mouse was inoculated into three weaned rats intraperitoneally (1·0 ml.) and three intracerebrally (0·1 ml.). All six remained well. Another two rats were inoculated intraperitoneally with 0·1 ml. of concentrated suspension of brain of a mouse of the 60th passage. Both became very weak in the hind legs at eight days, and remained so for four days, before gradually recovering.

Rabbits. Two rabbits were inoculated intraperitoneally with 2·5 ml. of a suspension of infected mouse brain (passage 72). There was no febrile response, and, when sacrificed at 29 days, a pool of organs from the two rabbits proved uninfective for mice. Complement fixing antibodies to the Innisfail strain were detected in the serum of one of the rabbits.

Chicks. Five 3-day-old chicks were inoculated intramuscularly with 0·03 ml. of an infective mouse brain suspension (passage 72). There were no signs of infection, and they were discarded at 23 days.

Histopathology of infections with the Innisfail strain.

Unfortunately, the organs of the water-rat were not available for examination.

Mice. The histopathological study of mice of the early passages (1-4) was made with the knowledge that they were also infected with *Toxoplasma.* However, as the histopathology of *Toxoplasma* infection in this strain of mice was recently studied (Pope *et al.*, 1957), it was possible to recognize the further distinctive lesions that were produced by the virus.

Tissues were examined from six paralysed mice of the first four passages, taken between four and fourteen days after inoculation. Three had been inoculated intraperitoneally, and three intracerebrally. The major abnormalities seen were in skeletal muscle and the central nervous system, while other organs examined showed no significant changes. The principal lesion in the muscles was a cellular infiltration, sometimes associated with swelling of fibres and loss of cross-striation. Examination of brain sections revealed mild meningitis, microglial infiltration and perivascular cuffing, common in *Toxoplasma* infection in mice, but there were also other lesions which were quite distinct from those produced by *Toxoplasma.* These included degeneration of neurons and focal granular necrosis.

Tissues of six mice of the 72nd-73rd passages, taken 2-3 days after inoculation, were also studied. These mice were infected with Innisfail virus alone, *Toxoplasma* having been eliminated. Little difference was observed histologically between the mice inoculated intraperitoneally or intracerebrally. Compared with the mice of the early passages, the degeneration of neurons was similar, but cellular reaction was lacking. Mild, focal, cellular infiltration of the myocardium was sometimes present. In contrast with the earlier passages, only focal lesions were observed in skeletal muscle. Liver, spleen, kidney and lung appeared normal.

Guinea-pigs. Lesions were seen in heart and brain of four infected guinea-pigs examined 5-9 days after inoculation with a suspension free of *Toxoplasma* (passage 71). Large areas of partly destroyed muscle fibres, with cellular infiltration, were scattered in the myocardium. There was some damage to neurons in the cerebrum, as well as small inflammatory lesions, perivascular cuffing, and meningitis. Cerebellum, liver, spleen, kidney and lung appeared normal.

Rats. Three laboratory rats were inoculated intraperitoneally with infective material, free from *Toxoplasma,* from mice of the 71st passage. One was sacrificed for examination at eight days. In brain sections, there were several focal inflammatory lesions, perivascular cuffing, and necrosis of some neurons. The second rat was examined at 13 days, and perivascular cuffing and degeneration

of neurons were also seen. Other tissues of these two rats, and all the tissues of the third rat examined at 37 days, appeared normal.

Identification of the Innisfail strain.

The properties of the Innisfail strain in mice, namely, pathogenicity, paralysis in mice surviving more than a few days, and presence of myositis and encephalitis with typical degeneration of neurons, were similar to those reported for the EMC group. An immunological study was therefore made of the relationship between the Innisfail strain and the EMC virus of Helwig and Schmidt.

TABLE 2.

Cross-neutralization between Innisfail and EMC viruses.

Virus	Antiserum	Virus dilution						Log LD_{50} dilution	Neutralization index log
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
Innisfail	Normal control (Rat 52)			6/6*	6/6	1/6	1/6	6·7	
	Innisfail (Rat 65)	6/6	3/6	4/6	0/6			4·8	1·9
	EMC (Rat 71)	6/6	3/6	1/6	1/6			4·3	2·4
EMC	Normal control (Rat 52)			6/6	6/6	5/6	2/6	7·6	
	Innisfail (Rat 65)	6/6	5/6	0/6	1/6			4·5	3·1
	EMC (Rat 71)	4/6	4/6	0/6	0/6			4·0	3·6

* Proportion of mice dead in each group.

The results of the cross-neutralization tests are summarized in Table 2. Rat antisera prepared against the Innisfail and EMC viruses neutralized significant amounts of each virus.

Rat antisera prepared against Innisfail and EMC viruses were also found to possess complement fixing antibodies to both antigens (Table 3).

TABLE 3.

Cross-complement fixation tests (reciprocals of titres) with the Innisfail and EMC viruses.

Antigen	Antisera		Normal rat serum control
	Innisfail	EMC	
Innisfail	32	32	0
EMC	32	32	0

It seems reasonable to conclude that the Innisfail strain is a typical member of the EMC group, on the basis of its characteristic properties, and the close immunological relationship to a known strain shown in these tests.

No neutralizing antibodies to the Innisfail virus were detected in the serum of the original *Hydromys*; possibly the infection was in the acute stage at the time of autopsy.

DISCUSSION.

An interesting property of the Innisfail strain is the different tissue tropism shown in mice and guinea-pigs. In infected mice, lesions of the central nervous system and skeletal muscle were severe, and cardiac lesions minimal; whereas in guinea-pigs, the macroscopic myocarditis somewhat overshadowed the encephalitis. The Columbia-SK virus apparently did not produce noticeable myocarditis in mice (Jungeblut and Sanders, 1940), while neither mice nor guinea-pigs infected with the Mengo virus showed myocarditis (Dick, 1948). On the other hand, mice, guinea-pigs and rabbits inoculated with EMC virus showed myocarditis (Helwig and Schmidt, 1945), as did also field rats and mongooses (Kilham *et al.*, 1956a, b). Such variations in production of myocarditis do not, however, detract from the close relationship within the EMC group.

Dick, Smithburn and Haddow (1948) recorded the isolation of Mengo virus from mosquitoes, *Taeniorhynchus fuscopennatus*, in Uganda; a finding that suggested the possibility of arthropod transmission. However, attempts at experimental transmission by *T. fuscopennatus*, *T. uniformis*, *T. africanus*, *T. perturbans* or *Aedes aegypti* failed (Dick, 1953; Vanella *et al*, 1956). As infected rodents excreted virus for some time, and experimental infection was successful by the oral route and by contact, it was suggested that contamination of food may be a natural method of transmission (Kilham *et al.*, 1956a; Vanella *et al.*, 1956). The long survival of the virus *in vitro* (Dick, 1948; Bingel and Engelhardt, 1953) would facilitate oral transmission.

The isolation of the Innisfail strain extends the known geographical distribution of the EMC group of viruses (Table 1), and the possibility of human infection in Australia must be considered. It is planned to make a survey of antibodies in human sera in north Queensland, paying particular attention to cases of undiagnosed fever.

Acknowledgments. The author wishes to thank Dr. E. H. Derrick for advice, and Dr. I. M. Mackerras for criticism of the manuscript. Thanks are also due to Mr. W. Scott for assistance with the animal studies and to Mr. G. M. Smith for the histological preparations.

REFERENCES.

Bieling, R. (1952): Wien. med. Wschr., 102, p. 106. Abstract in Excerpta Medica (1952), Sect. IV, 5, p. 1125.

Bingel, K. F., and Engelhardt, H. (1953): Z. Hyg. InfektKr., 138, p. 108. Abstract in Excerpta Medica (1954), Sect. IV, 7, p. 935.

Derrick, E. H., Pope, J. H., Chong, S. K., Carley, J. G., and Lee, Patricia E. (1954): Austral. J. exp. Biol., 32, p. 577.
Dick, G. W. A. (1948): Brit. J. exp. Path., 29, p. 559.
Dick, G. W. A. (1953): Trans. R. Soc. trop. Med. Hyg., 47, p. 13.
Dick, G. W. A., Best, A. M., Haddow, A. J., and Smithburn, K. C. (1948): Lancet, 2, p. 286.
Dick, G. W. A., Smithburn, K. C., and Haddow, A. J. (1948): Brit. J. exp. Path., 29, p. 547.
Helwig, F. C., and Schmidt, E. C. H. (1945): Science, 102, p. 31.
Hofman, B., Jr., and Jungeblut, C. W. (1952): Klin. Wschr., 30, p. 1013. Abstract in Excerpta Medica (1953), Sect. IV, 6, p. 472.
Horgan, E. S. (1951): Cited in "Viral and rickettsial infections of man", edited by Rivers, T. M., 2nd edition (1952), p. 678.
Jungeblut, C. W., and Dalldorf, G. (1943): Amer. J. publ. Hlth., 33, p. 169.
Jungeblut, C. W., and Sanders, M. (1940): J. exp. Med., 72, p. 407.
Keller, W., and Vivell, O. (1952): Klin. Wschr., 30, p. 1015. Abstract in Excerpta Medica (1953), Sect. IV, 6, p. 472.
Kilham, L., Mason, P., and Davies, J. N. P. (1956a): Amer. J. trop. Med. Hyg., 5, p. 647.
Kilham, L., Mason, P., and Davies, J. N. P. (1956b): *Ibid.*, 5, p. 655.
Kissling, R. E., Vanella, J. M., and Schaeffer, M. (1956): Proc. Soc. exp. Biol., N.Y., 91, p. 148.
Lennette, E. H., Clark, W. H., and Dean, B. H. (1949): Amer. J. trop. Med., 29, p. 527.
Pope, J. H., Derrick, E. H., and Cook, I. (1957): Austral. J. exp. Biol., 35, p. 467.
Reed, L. J., and Muench, H. (1938): Amer. J. Hyg., 27, p. 493.
Roca-Garcia, M., and Sanmartin-Barberi, C. (1957): Amer. J. trop. Med. Hyg., 6, p. 840.
Smadel, J. E., and Warren, J. (1947): J. clin. Invest., 26, p. 1197.
Tiggelman-van Krugten, V. A. H., and Collier, W. A. (1955): Doc. Med. geog. trop., 7, p. 270.
Vanella, J. M., Kissling, R. E., and Chamberlain, R. W. (1956): J. infect. Dis., 98, p. 98.
Verlinde, J. D., and Molron, J. H. (1954): Leeuwenhoek ned. Tijdschr., 20, p. 129.
Verlinde, J. D., and van Tongeren, H. A. E. (1953): Arch. Ges. Virusforsch., 5, p. 217. Abstract in Excerpta Medica (1954), Sect. IV, 7, p. 935.
Warren, J. (1952): In "Viral and rickettsial infections of man", edited by Rivers, T. M., 2nd edition (1952), p. 675.
Warren, J., Russ, Sudie B., and Jeffries, Helen (1949): Proc. Soc. exp. Biol., N.Y., 71, p. 376.
Warren, J., Smadel, J. E., and Russ, Sudie B. (1949): J. Immunol., 62, p. 387.

THE EFFECT OF OPERATION AND CHRONIC LYMPH FISTULA ON PRODUCTION OF TISSUE FLUID AND LYMPH[1]

by G. WOOLLEY[2] AND W. J. SIMMONDS

(From the Department of Physiology, University of Western Australia).

(*Accepted for publication 14th November, 1958.*)

SUMMARY.

In unanaesthetized rats, successive daily changes in flow and composition of lymph with only slow, continuous intragastric infusion of saline have been studied. In some cases, responses to additional intravenous or intragastric infusion for 7 hr. each day were investigated. Body weight was measured continuously in all rats without interfering with any of the experimental procedures.

In all rats there was a progressive fall in body weight and a considerable fall in concentration of protein in the circulating plasma during 4 days following operation to establish the lymph fistula. In some rats, whether extra infusions were subsequently given or not, lymph flow under basal conditions was lower on the first day than during a corresponding period on the second day. Reasons are given for ascribing this to a depression of capillary circulation on the first post-operative day. Protein concentrations in pre-infusion lymph fell in rats which received repeated infusions. This did not occur in rats which had no extra infusions, and in such rats an improvement in lymph flow on the second post-operative day was accompanied by a comparable increase in protein output in lymph.

The response to intravenous infusion did not alter materially as the days went by. Intragastric infusion produced a smaller increment in lymph flow on the first post-operative day than subsequently. On the second and third days intragastric infusion, as expected, produced a greater increase in lymph flow than the same dose intravenously. In addition, intragastric infusion washed out more protein from tissue spaces into lymph than did intravenous infusion under the conditions of these experiments.

INTRODUCTION.

Intestinal tissue fluid, the major source of thoracic duct lymph in unanaesthetized rats, is itself derived from two sources – fluid absorbed from the intestinal lumen and fluid filtered from the intestinal capillaries (Simmonds, 1954; Kim and Bollman, 1954). In previous experiments, the effect of increased fluid absorption has been studied and the mechanism of the increased capillary filtration and lymph flow during fat absorption has been investigated (Simmonds,

[1] This work was carried out with the aid of grants from the National Health and Medical Research Council, Canberra, and the Medical Research Grants Committee, University of Western Australia.

[2] Technical Officer (Science) of the National Health and Medical Research Council.

1954, 1957). In the experiments reported below, changes in the formation and composition of lymph following an operation (cannulation of abdominal thoracic duct and insertion of a gastrostomy tube) have been investigated, starting on the first day after operation and continuing for four successive days. Changes in the capillary circulation, in plasma protein concentration, in bodily hydration and in absorption of fluid from the intestines might be expected as a result of the operation and of losses through the lymph fistula. The object of the experiments was to determine the effect of such changes on the formation of tissue fluid and lymph. In some experiments Ringer-Locke solution was infused intravenously, to enhance capillary filtration and amplify the effects of post-operative changes in this aspect of tissue fluid formation. On other occasions, extra fluid was given instead by the intragastric route, but at the same rate, to emphasize any changes in contribution by absorbed saline to tissue fluid formation and to compare the effects of infusion by the two routes. Changes in bodily hydration were followed by continuous weighing. It was also hoped that such experiments would provide a useful background to subsequent work on the effect of vasoactive substances on visceral capillary circulation and lymph formation.

METHODS.

Male rats of hooded strain weighing from 180 to 220 gm. were used. The thoracic duct and right jugular vein were cannulated and a rubber-tipped gastrostomy tube inserted, as previously described (Simmonds, 1957). Animals were placed in a restraint cage of a similar type to that designed by Bollman (1948), which was mounted on one arm of a Towers two pan balance weighing to 0·1 gm.

After recovery from anaesthesia, intragastric infusion of Ringer-Locke solution at 0·6 ml. per hour was commenced, using a constant infusion roller pump. This infusion was continuous until the end of the experiments (up to 5 days) except during experimental infusions. These were made from a second pump for periods of 7 hours at a rate of 2·6 ml. of saline per hour given either intravenously or intragastrically, after warming to body temperature. Experimental infusions were not commenced until the first post-operative day (20 to 24 hours after operation) nor were they commenced unless the flow of lymph was definitely unobstructed and the clinical condition of the rat satisfactory. An experimental session was begun usually at 9 a.m. and the lymph flow and changes in weight measured hourly. When two successive hours showed a steady lymph flow, infusion at 2·6 ml. per hour was commenced either intravenously or intragastrically. This was continued for at least 7 hours. Hourly lymph samples were collected and urine flow and change in weight of the animal recorded hourly. Lymph protein concentration was determined subsequently by the biuret method (Gornall, Bardawill and David, 1949) modified to include an ether washing procedure of the final reaction mixture and blanks. The ether washing was necessary to remove fat and hence reduce turbidity of the reaction mixture.

RESULTS.

Post-operative changes in lymph formation.

Fig. 1 shows the average results in 9 rats used for this purpose. Five of these received an additional infusion each day, Fig. 1(b). However, the infusion

commenced immediately *after* the period for which results are plotted and was completed at least 14 hr. before the pre-infusion observations on the following day. To demonstrate any residual effects of repeated infusions another 4 rats, Fig. 1(a), were given only the routine intragastric supplement, 0·6 ml./hr., throughout the entire post-operative period.

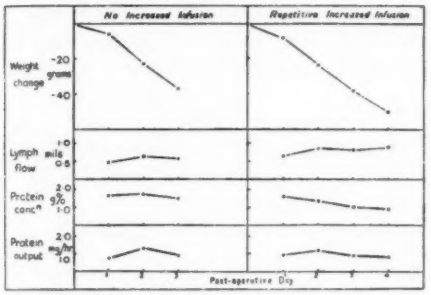


Fig. 1. Weight changes on successive post-operative days in four rats receiving steady Ringer-Locke saline infusion at 0·6 ml./hr. and nine rats receiving additional 7 hour infusions at 2·6 ml./hr. subsequent to the daily 2 hour periods from which this figure is plotted. Mean lymph flow, protein concentration and protein output for the two hours preceding increased infusion or over the corresponding two hours in rats receiving only steady slow infusion.

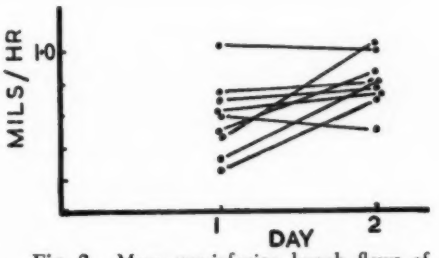


Fig. 2. Mean pre-infusion lymph flows of individual rats on first and second post-operative days. (See text.)

Body weight fell progressively in all rats. Weight loss was usually less during the first 24 hr., due probably to clotting of lymph at frequent intervals during this period and to post-operative depression of urine flow. There were no significant differences between weight loss in the two groups, Fig. 1(a) and (b). Although 3-4 gm. of fluid remained at the end of each infusion in animals which received extra fluid, Fig. 1(b), they did not retain it, despite a steadily increasing dehydration as days went by.

Plasma protein concentrations fell considerably. Serial estimations are not available but in five rats with extra infusions the protein concentration fell from 5·6 to 3·5 gm./100 ml. in an average of 92 hr. In three rats which received only the basal intragastric fluid the concentration fell from 5·6 to 3·7 gm./100 ml. in an average of 92 hr.

Lymph flow showed considerable individual variation. Variation between rats was significantly greater on the first post-operative day than on the 2nd and 3rd. There was a significant increase in mean flow between 1st and 2nd days which, in individual rats, was seen only in conjunction with a low rate of flow on the first day. This is illustrated by Fig. 2.

Protein concentrations in lymph fell progressively in the group which received additional infusions, Fig. 1(b), but not in the other group. This difference in response was statistically significant ($P < 0{\cdot}05$ in analysis of variance).

Protein output in lymph showed no significant trend in the infused group, Fig. 1(b), but increased in non-infused rats between 1st and 2nd post-operative days. This difference in behaviour was not statistically significant but was consistent with the significant increase in lymph flow seen in both groups between 1st and 2nd days and the significant decrease in protein concentration in lymph in infused rats but not in those which received no additional infusions.

Response to fluid loading.

With intravenous infusions, as Fig. 3 shows, the pattern of response was much the same from 1st to 4th post-operative days. In nearly all cases lymph flow increased progressively during the first 4 hr. Thereafter it altered very little, remaining at a level which varied in different animals from 1·4 to 2·8 times the rate before infusion. There was no consistent alteration in response as the days went by. On the 2nd, 3rd and 4th, but not on the 1st post-operative day, the fall in protein concentration in lymph lagged somewhat behind the increase in lymph flow and reached a new equilibrium value more slowly. In most cases, however, protein concentrations were steady during the last 2 hr. of infusion.

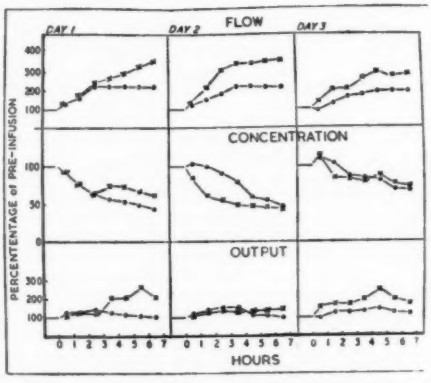


Fig. 3. Lymph flow, protein concentration and protein output as percentages of the mean pre-infusion levels on a given day in rats receiving 2·6 ml./hr. of Ringer-Locke saline.
● — ● Intravenous infusion.
× — × Intragastric infusion.

With intragastric infusion, the lymph flow increased more rapidly and the protein concentration fell more abruptly on the 2nd post-operative day than on the 1st.

The route of infusion had a considerable effect on the response as can be seen in Fig. 3 and Tables 1 and 2. Fig. 3 shows that intragastric infusion produced the greater increase in lymph flow, expressed as percentage of pre-infusion rate of flow. Table 1 shows that more of the added fluid appeared as lymph after intragastric infusion. The weight changes indicated that intragastric infusion produced a greater lymph flow with a smaller retained load of fluid. In fact, on the 2nd and 3rd post-operative days a high lymph flow was sustained by intragastric fluid despite a decreasing body weight during the latter hours of infusion. Intragastric fluid washed more protein out of tissue spaces into lymph during the 7 hr. infusion (Table 2), except on the 2nd post-operative

day when the amount of protein lost in excess of that expected from the pre-infusion output was the same for both routes. However, with intragastric infusion the lymph collected during the 7 hr. period did not account for the total amount of protein washed out of tissue fluid. As can be seen from Fig. 3, with intragastric infusion the protein output during the 7th hr. was still considerably greater than before infusion. With intravenous infusion, on the other hand, protein "washout" was apparently complete by the 7th hr., the increased lymph flow being then balanced by a proportionate decrease in protein concentration so that protein output in lymph was once more the same as before infusion.

TABLE 1.

Influence of infusion route on weight change and lymph flow.

Treatment	Day No.	No. of rats	Wt. change grams		
			Hours 0-4	Hours 4-7	Total
I.V. Infusion 2·6 ml/hr.	1	3	2·3	2·2	4·5
	2	5	3·3	0·7	4·0
	3	3	3·5	0·9	4·4
	4	4	2·2	0·6	2·8
I.G. Infusion 2·6 ml/hr.	1	3	2·8	1·2	4·0
	2	4	2·6	−1·4	1·1
	3	2	4·1	−2·1	1·4
			Excess lymph flow*		
			Hours 0-4	Hours 4-7	Total
I.V. Infusion 2·6 ml/hr.	1	3	2·2	2·3	4·5
	2	5	2·0	2·5	4·6
	3	3	1·3	2·4	3·7
	4	4	1·7	2·2	3·8
I.G. Infusion 2·6 ml/hr.	1	3	1·4	3·6	5·1
	2	4	3·0	4·1	7·1
	3	3	2·7	3·4	6·1

* Excess lymph flow: the observed lymph flow less the mean hourly pre-infusion flow ×4 or 3.

TABLE 2.

Excess protein output during 7 hr. infusion period.*

Day	No. of rats	I.V. 2·6 ml/hr.	No. of rats	I.G. 2·6 ml/hr.
1	3	17·5 mg.	3	57·6 mg.
2	5	21·4 mg.	4	20·5 mg.
3	3	18·9 mg.	2	39·8 mg.
4	4	11·5 mg.		

* Excess protein output: the observed protein output less the mean hourly pre-infusion output ×7.

DISCUSSION.

Three aspects of the results merit brief discussion. Firstly, there were changes in lymph formation during what will be called, for brevity, the "basal" periods—i.e. the period each morning during which no extra infusion was given and when at least 14 hr. had elapsed since any preceding infusion. Secondly, the effect of infusions on lymph formation altered as days went by, and thirdly, the route of infusion, intravenous or intragastric, influenced the results.

The "basal" lymph flow was often greater on the second day after operation than on the first. This could be interpreted either as a depression of lymph formation on the first day or as an enhancement on the second. The former would presumably be a consequence of the preceding operation. The latter could be an after-effect of the infusion on the first day or due to hypoproteinemia or other progressive effects of the lymph fistula. A post-operative restriction of capillary circulation and lymph production, persisting into the first day, seems more likely for a number of reasons. Most cogent, perhaps, was the relative uniformity in flow rates between rats on the second day and the absence of a change between second and third days. It was only on the first day that a considerable variation occurred between rats, and this was due to a number of animals in which flow rates were less than 0·5-0·6 ml./hr. It was only these rats which subsequently showed a considerable increase in lymph flow, by 50-100 p.c., between 1st and 2nd days (see Fig. 2). Their lymph flows were thereafter comparable with those in the other rats, which showed little alteration during the first three days. There was no indication that diminished flow on the first day was due to overt shock, illness or partial obstruction of the cannulae. Measurements of protein output in the lymph in a group of non-infused rats also favours the idea of a restricted number of functioning capillaries in animals with slow lymph flow on the first day. The subsequent increase in lymph flow was not accompanied by a substantial drop in protein concentration in the lymph and so there was a considerable increase in output of protein as well as of fluid from the fistula. An increase in filtration through the same set of capillaries, due to hypoproteinemia or raised capillary pressure, would lead to a considerable lowering of the concentration of protein in tissue fluid and so in lymph. An increased lymph flow without much alteration in protein concentration, as we obtained, is more consistent with an increase in the number of functioning capillaries. This would increase the volume of tissue fluid and the turnover of lymph without necessarily altering the equilibrium concentration of protein in the tissue spaces. The absence of changes between second and third days would not support a progressive increase in capillary permeability as an alternative explanation of the findings.

Repeated infusions had little effect, *per se*, on lymph flow in successive "basal" periods. In rats which received no additional infusions the pattern of changes in flow from day to day was the same, although the actual flows were

somewhat lower on all three days, whether expressed as ml./hr. or ml./kg./hr. However, infusions washed extra plasma protein out of tissue spaces and this was not completely replaced before the next "basal" period, 14 hr. later. This was shown by the progressive fall in lymph protein concentration which occurred only in the rats which received daily infusions. Protein concentrations in the circulating plasma fell to about the same extent in the first 90 hr. after operation in rats which were repeatedly infused as in those which were not. This raises a point of some interest. It implies that the difference in protein concentration between plasma and tissue fluid was less in non-infused rats, which showed higher concentrations in tissue fluid and lymph. That is, the colloidal osmotic pressure difference opposing filtration was less in this group although, as mentioned above, the turnover of tissue fluid and lymph was slower. Possibly hydrostatic filtering pressure in capillaries was lower in the absence of repeated infusions – although the weight changes did not suggest that such extra fluid was retained more than a few hours. More likely, fewer capillaries were open for filtration.

Comparing the response to intravenous infusion on successive post-operative days, there were no striking differences. More experiments would be required to determine whether rats with a low basal lymph flow on the first day after operation responded differently from the others. With intragastric infusions there was evidence of slower intestinal absorption of fluid on the first day after operation, since the lymph flow increased and protein concentration in lymph fell more slowly during the first infusion than on subsequent days.

The response to fluid loading by the alternative methods, intragastric or intravenous infusion, was best compared on second and third days after operation. By that time any post-operative depression of lymph production and intestinal absorption had passed off. It was found that an intragastric infusion stimulated lymph flow more than the same fluid load by intravenous injection. This was not unexpected since intragastric infusion delivers the fluid, after absorption, to the chief source of thoracic duct lymph, viz. intestinal tissue fluid. After intravenous infusion, on the other hand, the fluid load is distributed to all tissues, the intestinal tissue spaces receiving only their share. Kim and Bollman (1954) found that the total volume of lymph produced during three days' continuous intragastric infusion varied with the composition of fluid infused and its ability to maintain the total extracellular volume after absorption. In the shorter infusion periods studied by us, a stage was reached towards the end of infusion when total body weight was declining but lymph flow remained steadily elevated. Under these conditions, and with single doses of fluid (Simmonds, 1954), the local expansion of intestinal tissue fluids by fluid in course of absorption seemed to be the essential mechanism. It may be that the decline in body weight merely reflected the onset of diuresis and expulsion of urine progressively accumulated in the bladder. Arrangements for urine collection

were not satisfactory enough to enable this objection to be overruled. Our findings do not, of course, invalidate Kim and Bollman's interpretation of the results of prolonged infusion. Benson, Lee, Scholer, Kim and Bollman gave a single injection of D_2O during the course of continuous infusion. They recovered very little of the label in lymph during the subsequent hour whether D_2O was given by intravenous or intragastric routes. They also demonstrated very rapid equilibration of isotope concentrations between plasma, tissue fluid and lymph. Such equilibration, however, would go on independently of volume changes in tissue fluid. It would not measure the slower bulk transfer of fluid from intestinal lumen to tissue spaces and thence to both capillaries and lymphatics. While the individual water molecules swelling lymph flow have participated in exchange by diffusion, a substantial part of the extra volume of lymph recovered during intragastric infusion may still be regarded as drainage of absorbed fluid from the tissue spaces. Whether the volume of absorbed fluid passing by this pathway is as large in an intact animal as with free drainage from a lymph fistula it is difficult to say.

Acknowledgments. We wish to thank Mr. N. S. Stenhouse of the C.S.I.R.O. for statistical analysis of the results, and Miss H. Buchan for technical assistance.

REFERENCES.

Benson, J. A., Jr., Lee, P. R., Scholer, J. F., Kim, K. S., and Bollman, J. L. (1956): Amer. J. Physiol., 184, p. 441.
Bollman, J. L. (1948): J. lab. clin. Med., 33, p. 1348.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949): J. biol. Chem., 177, p. 751.
Kim, K. S., and Bollman, J. L. (1954): Amer. J. Physiol., 179, p. 273.
Simmonds, W. J. (1954): Austral. J. exp. Biol., 32, p. 285.
Simmonds, W. J. (1957): Quart. J. exp. Physiol., 42, p. 205.

FACTORS IN THE COLORIMETRIC ESTIMATION OF 17-KETOSTEROIDS IN URINE[1]

by JUNE B. SHEATH

(From the Diabetic and Metabolic Unit, Alfred Hospital, Melbourne).

(*Accepted for publication 11th November, 1958.*)

SUMMARY.

A method has been described for the colorimetric estimation of urinary 17-ketosteroids.

Although similar to conventional techniques, the method has been modified in a number of ways such as changes in the conditions for the development of colour in the Zimmermann reaction.

It has been shown that the use of formalin prior to hydrolysis obviates the necessity for the use of a correction formula.

INTRODUCTION.

The determination of neutral urinary 17-ketosteroids by colorimetric means was first suggested by Callow, Callow and Emmens (1938). The method was derived from a reaction for the determination of ketonic substances originally described by Zimmermann (1935, 1936). This reaction which bears his name has formed the basis for a variety of methods which have been suggested for the estimation of these steroids (Langstroth and Talbot, 1939; Talbot, Butler and MacLachlan, 1940; Holtorff and Koch, 1940; Fraser, Forbes, Albright, Sulkowitch and Reifenstein, 1941; Cahen and Salter, 1944; Vestergaard, 1951; Drekter, Heisler, Scism, Stern, Pearson and McGavack, 1952). It is obvious from the number of procedures recommended that no single one is entirely satisfactory.

The purpose of this investigation has been two-fold: Firstly, to examine some of the methods of 17-ketosteroid estimation with particular reference to:

(a) Hydrolysis of urine.
(b) Purity of reagents.
(c) The optimal times and temperatures for the Zimmermann reaction.
(d) The analysis of absorption spectra of pure steroids and ketonic and other urinary extracts.

Secondly, to compare results obtained by the use of a correction formula for interfering chromogens with those of a simple procedure for 17-ketosteroid determination which obviates the need for such a formula.

---

[1] This paper was portion of the work submitted for the degree of Master of Science, Melbourne University.

Methods.

Reagents.

(a) Concentrated hydrochloric acid (A.R., B.D.H.) is used in all determinations.

(b) Formaldehyde, 40 p.c. C.P.

(c) Ether. Anaesthetic grade, purified by standing over potassium hydroxide for several days. It is then fractionally distilled and the fraction boiling between 34° and 35° C. is collected and stored over saturated ferrous sulphate.

(d) Sodium hydroxide (A.R., B.D.H. pellets), aqueous, approximately 20 p.c.

(e) Ethanol (A.R., B.D.H.) is used for the Zimmermann reaction. For the dilution of samples after the Zimmermann reaction purified commercial ethanol is used. This is prepared by fractionally distilling ethanol which has been allowed to stand over potassium hydroxide pellets for several days and subsequently collecting the fraction boiling at 78° C.

(f) m-dinitrobenzene. This is purified by gently warming 500 ml. of 0·1N sodium hydroxide to which approximately 15 grams of m-dinitrobenzene have been added. After cooling, the precipitate of m-dinitrobenzene is collected by filtration and dissolved in a minimal volume of warm ethanol. Crystalline m-dinitrobenzene, precipitated by the addition of a large volume of distilled water to the alcoholic solution, is recovered by filtration. It is then washed several times with distilled water until the washings are neutral. This material is twice recrystallised from ethanol, dried in a vacuum desiccator and stored in a dark glass-stoppered bottle. The melting point of the final product is between 89° and 90° C.

For the Zimmermann reaction a freshly prepared 2 p.c. ethanolic solution of m-dinitrobenzene is used.

(g) Potassium hydroxide pellets (A.R., B.D.H.).

(h) Methanol. A.R. grade methanol is purified by fractional distillation from a saturated solution of phenylhydrazine hydrochloride. The fraction distilling at 74° C. is redistilled twice.

(i) 2·5N methanolic potassium hydroxide (modified from Krieger, 1950). Approximately 8 grams of potassium hydroxide pellets are transferred to a 50 ml. measuring cylinder chilled by ice-water. Redistilled methanol is added to a volume of 50 ml., and after vigorous stirring, the solution and the carbonate precipitate are transferred to a glass-stoppered flask. This is then filled with nitrogen and stored in the cold for 4-7 days. After centrifugation at 4° C. to remove carbonate, the supernatant is quickly transferred to a glass-stoppered flask. The solution is standardised by titration of 1 ml. against 0·1N hydrochloric acid with methyl orange as indicator, and its normality adjusted to within the limits of $2{\cdot}50 \pm {\cdot}02$. This solution, stored under nitrogen at 4°-6° C. is stable for many weeks. Deterioration is indicated by an increase in optical density or slight precipitation of the reagent blank.

(j) 2·5N ethanolic potassium hydroxide is prepared in the same manner as the methanolic solution, but the preparation and standardisation are performed on the day of the test.

(k) Girard's reagent T (laboratory reagent grade).

(l) Glacial acetic acid (A.R., B.D.H.).

(m) Sodium hydroxide (A.R., B.D.H. pellets), aqueous, approximately 10 p.c.

(n) Sodium carbonate (A.R., B.D.H.), aqueous, 2·5 p.c.

(o) Standards. Pure androsterone and dehydroisoandrosterone are used as standards. Stock ethanolic solutions of 250 mg./100 ml. when stored at 4° C. are found to be stable indefinitely. For working standards, these are diluted with A.R. ethanol to 5 mg./100 ml. immediately prior to use.

Technique.

(a) All glassware used for the estimation must be treated with chromic acid, extensively washed in tap water and finally rinsed in distilled water.

(b) Urine collection. Twenty-four hour specimens of urine must be collected from patients into glass bottles containing 10 ml. of concentrated hydrochloric acid. Convenient (250 ml.) aliquots are stored in the cold prior to estimation. It has been found that samples of urine may be stored for periods up to three weeks with no appreciable loss of 17-ketosteroids.

(c) Acid hydrolysis. 7·5 ml. of hydrochloric acid are added to 50 ml. of urine, the mixture is then simmered for 15 minutes and cooled in ice-water and is then kept in a stoppered flask.

(d) Acid hydrolysis in the presence of formalin (Antunes, 1956). To 50 ml. of urine is added 0·15 ml. of 40 p.c. formaldehyde followed by 7·5 ml. of hydrochloric acid and the mixture hydrolysed as in (c).

(e) Extraction. The urine, hydrolysed either in the presence or absence of formalin, is extracted by repeated shaking with 100 ml. of ether and re-extracted with two 50 ml. aliquots of ether. Phenolic compounds and some pigment are then removed by washing the combined ether extracts twice with 50 ml. of 20 p.c. sodium hydroxide. Minimal volumes of distilled water are used to wash the extract until the washings are neutral to litmus. The extract is transferred quantitatively to a conical flask and the ether removed by distillation from a hot water bath. Following the addition of 3 ml. of absolute ethanol, the extract is dried completely at room temperature under reduced pressure.

After drying the extract is dissolved in 10 ml. ethanol. Suitable aliquots (1 ml.) of this solution are transferred to glass-stoppered test tubes and evaporated almost to dryness in a hot water bath, the last traces of ethanol being removed under reduced pressure.

(f) Girard fractionation. The micro technique of Pincus and Pearlman (1941) has been used throughout.

(g) 17-ketosteroid standards. These are prepared by introducing measured amounts of an ethanolic solution of pure 17-ketosteroid into reaction tubes and drying them in the same manner as urine extracts. All tubes containing standards or urine extracts are stoppered and stored in the cold prior to the Zimmermann reaction.

(h) Zimmermann reaction. A reagent blank, a set of standards and the urine extracts are prepared. To each tube is then added 0·2 ml. of each of the following: A.R. ethanol, 2 p.c. ethanolic m-dinitrobenzene and 2·5N methanolic potassium hydroxide. These reagents are thoroughly mixed in subdued light and incubated in a darkened water-bath at 25° C. After 45 minutes the reaction is terminated by cooling the tubes in an ice-water mixture. 5 ml. of distilled ethanol are added to bring the preparation to a volume suitable for colorimetric estimation.

The colour intensities of the individual specimens are read within 10 minutes of dilution in a Klett-Summerson null-point photoelectric colorimeter with the No. 54 (green: 500-570 mμ) and the No. 42 (blue: 400-465 mμ) filters.

RESULTS.

(a) *Hydrolysis of urine.*

Using the technique described, the effect of acid hydrolysis on certain 17-ketosteroids has been investigated. Known amounts of both androsterone and dehydroisoandrosterone in ethanol were added to urine both before and after acid hydrolysis. Table 1 shows that acid hydrolysis is associated with a considerable loss of dehydroisoandrosterone and with some loss of androsterone when compared with the almost complete recovery of both steroids if added to urine after acid hydrolysis. It has been shown elsewhere that a greater number

TABLE 1.

The effect of acid hydrolysis on the recovery of added free steroids.

Steroid	No. of expts.	Mean p.c. recovery	Range
(a) Androsterone	3	75	72-81
(a) Dehydroisoandrosterone	5	31	23-53
(b) Androsterone	5	91	81-100
(b) Dehydroisoandrosterone	5	96	85-100

(a) 0·05 mg. steroid added to urine and then subjected to acid hydrolysis.
(b) 0·05 mg. steroid added to urine after acid hydrolysis.

of steroids can be recovered from urines subjected to enzymic hydrolysis with β-glucuronidase and subsequent acid hydrolysis, than when subjected to acid hydrolysis alone (Sheath, 1959).

(b) *Selection of conditions for Zimmermann colour reaction.*

(i) Optimal reaction temperature and time. The results of these observations suggest that 25° C. is a more suitable reaction temperature than 37° C., and that 45 minutes is the most satisfactory time for the development of colour.

Two reaction temperatures have been studied. At 37° C. it was found that pure steroids reached maximal colour development as measured by optical density between 30 and 50 minutes. With further incubation there was a decrease in optical density. Urine extracts reached a maximum in 30 minutes. Thereafter atypical colours and a decrease in optical density measurements were observed.

No qualitative colour changes were noticed if pure ketosteroids or urine extracts were incubated at 25° C. The colour increased gradually with the time of incubation and a linear rate of increase in colour intensity was observed for pure ketosteroids when incubated at 25° C. for 60 minutes. With urine extracts linearity was only preserved up to 45 minutes incubation. Therefore, the incubation time of 45 minutes was selected as being the most suitable. Under these conditions (45 minutes incubation at 25° C.) both pure ketosteroids and urine extracts were observed to obey Beer's Law.

(ii) Selection of filters. This was decided by the known wavelength maxima as determined by absorption curves on pure steroids, and ketonic and non-ketonic urine extracts. As has been shown by other workers the two most satisfactory filters for optical density measurement of crude urine extracts are the green (500-570 mμ) and the blue (400-465 mμ), because pure 17-ketosteroids show a single peak of absorption at 510-530 mμ and crude urine extracts show broad absorption bands in the region of the blue filter.

(iii) Methanolic v. ethanolic potassium hydroxide. At the commencement of these investigations difficulties were encountered with the use of 2·5N ethanolic potassium hydroxide. It was found that these solutions deteriorated

rapidly and gave much higher readings of blanks, standards and unknowns compared with methanolic potassium hydroxide. The latter was simple to prepare and gave lower blank and standard readings. Furthermore, if kept in the cold, it is stable for at least one month after preparation. With either solvent and pure ketosteroids, Beer's Law is obeyed over the range used in these experiments (0·0125 mg. to 0·25 mg.).

(c) *Absorption spectra.*

(i) Pure ketosteroids. The absorption spectra of several pure ketosteroids subjected to Zimmermann reaction are shown in Fig. 1. It will be seen that the

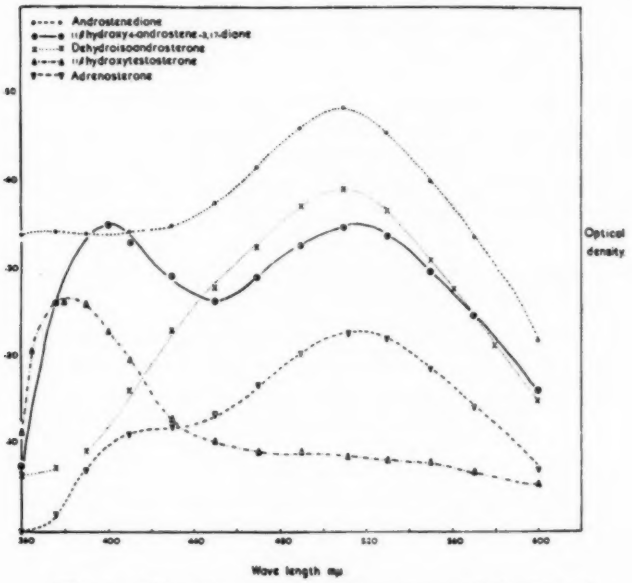


Fig. 1. Absorption spectra of Zimmermann derivatives of pure steroids.

absorption maxima for all these substances are in the 510-530 mμ region and that those steroids with oxygen functions at C_{11} show an additional absorption maximum in the 380-400 mμ region. The absorption spectrum of a mixture of pure steroids, dehydroisoandrosterone, androsterone, androstenedione, adrenosterone, 11, β-hydroxyandrostene-3,17-dione and 11, β-hydroxytestosterone has an absorption maximum at 520 mμ but also a small peak corresponding to the 380-400 mμ peaks shown by 11, β-hydroxyandrostene-3,17-dione and 11, β-hydroxytestosterone. In agreement with the findings of Landau (1949), different 17-ketosteroids have different chromogenic values. That of dehydroisoandro-

sterone is intermediate, and is a reasonable reference standard for comparison with the extract.

(ii) Ketonic urine extracts. Urine extracts subjected to Girard's fractionation and subsequent Zimmermann reaction show absorption spectra maxima in the 520 mμ region similar to those of pure steroids.

(iii) Non-ketonic extracts obtained from Girard fractionation. The non-ketonic extracts subjected to the Zimmermann reaction showed a maximal absorption at 330-370 mμ and a minimum between 480 and 560 mμ.

(d) *Extracts of formalin-treated urine.*

Extracts of urine to which formalin had been added prior to acid hydrolysis were constantly observed to be colourless. This agrees with the original observations of Antunes (1956).

The following investigations indicated that such treatment did not affect the chemical properties of 17-ketosteroids. The absorption spectra of pure ketosteroids recovered from water are unaltered by prior treatment with formalin (Fig. 2). Furthermore, the absorption spectra of urine extracts treated with formalin are virtually the same as their respective ketonic fractions (Fig. 3).

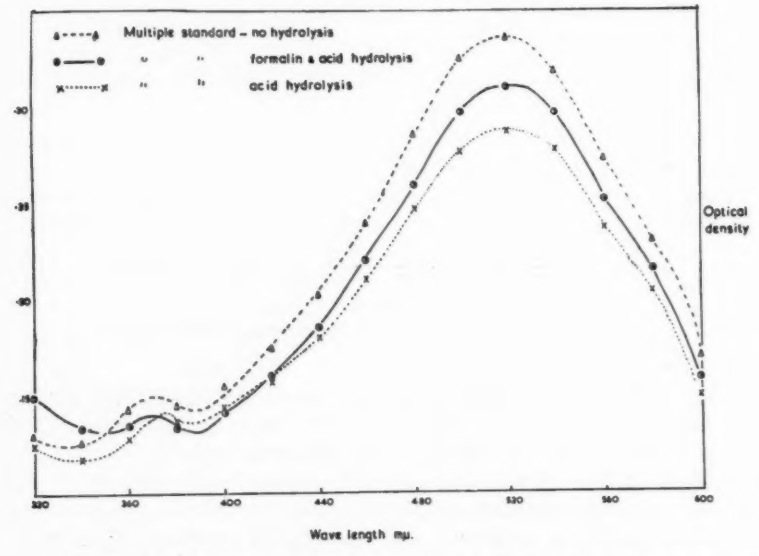


Fig. 2. Absorption spectra of Zimmermann derivatives of a multiple reference standard containing androstenedione, adrenosterone, androsterone, dehydroisoandrosterone, 11, β-hydroxy-4-androstene-3,17-dione and 11, β-hydroxytestosterone.

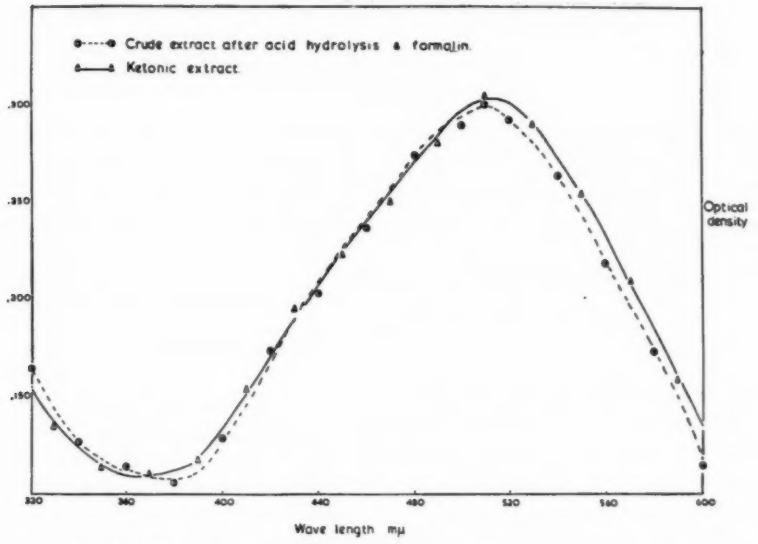


Fig. 3. Comparison of absorption spectra (Zimmermann reaction) of urine subjected to (a) formalin and (b) Girard fractionation.

All showed absorption maxima in the 510-530 mμ region.

Urine extracts subjected to chromatography in a petroleum-ether methanol-water descending system showed identical patterns with and without prior formalin treatment. A typical chromatogram is shown in Fig. 4.

(e) *Correction formulae.*

The application of the Zimmermann reaction to the determination of urinary 17-ketosteroids has posed an unsolved problem in the presence of coloured compounds in the crude urine extract. So far, the only method suggested to overcome this problem has been to subject the urine extract to Girard fractionation and determine the ketosteroid value by reading the ketonic fraction. This is a laborious procedure and one which does not readily lend itself to routine clinical use. In consequence, a variety of correction formulae have been devised to remove the labours of Girard fractionation.

Despite the introduction of such formulae most persons interested in the determination of urinary 17-ketosteroids are continually perplexed by the great variations in colour of the crude extracts of different urines which may range from pale brown to burgundy or deep blue. There is every reason to believe that these colours contribute in a substantial and varying degree to the final colour of the fully developed Zimmermann reaction. It is difficult to make compensation for these colours by the use of urine blanks. It is reasonable to

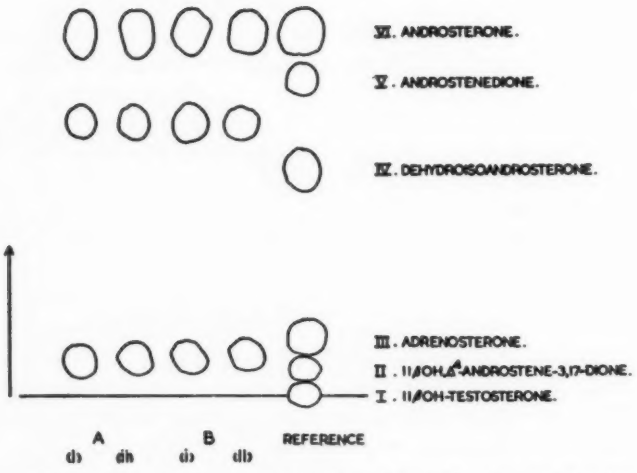


Fig. 4. Chromatogram of ketonic and formalin extracts of acid hydrolysed urines from subjects A and B. A (i) and B (i) are ketonic extracts. A (ii) and B (ii) are formalin extracts.

suspect that the application of the same correction formula to a urine extract that is highly coloured will lead to errors of under- or over-correction in the final estimation of 17-ketosteroids.

Nevertheless, most methods for the determination of 17-ketosteroids apply some form of correction formula, usually one requiring an additional reading of the sample with a blue filter. A formula which has been widely used is one recommended by the M.R.C. Committee on Clinical Endocrinology (1951). This formula was derived from the work of Talbot, Berman and MacLachlan (1942) who studied the amount of colour produced by ketonic extracts of urine and applied the theoretical correction for interfering pigments elaborated by Gibson and Evans (1937) and Gibson and Evelyn (1938). This correction was based upon Vierodt's theory that the respective concentrations of two pigments in solution may be measured provided the absorption peaks of the two pigments are known.

This formula proposed by Gibson and Evans (1937) and elaborated by Fraser *et al.* (1941) and Talbot *et al.* (1942) may be written:

$$G_c = \frac{K_1 (G_o) - B_o}{K_1 - K_s}$$

where K_1 is the ratio of blue to green readings of non-ketonic fractions and K_s the ratio of blue to green readings of pure steroids. These values were found to be 1·38 (K_1) from a series of 22 determinations and 0·64 (K_s) from a series of 115 determinations using dehydroisoandrosterone as the pure steroid. If these values are applied to the Gibson and Evans equation, the following correction formula is obtained for the method of 17-ketosteroid determination that has been described:

$$G_c = \frac{1{\cdot}38\ G_o - B_o}{0{\cdot}74}$$

It is felt that this formula is open to certain criticisms. Firstly, although the absorption peak of pure 17-ketosteroids and ketonic urine fractions is well defined (510-530 mμ), that of the non-ketonic fraction obtained from crude urine extracts is extremely broad and often poorly defined. Secondly, the application of this formula to the "green" and "blue" readings obtained from the urine of adrenalectomised oophorectomised females results on occasions in low normal values for steroid excretion, where none should be present. This suggests that the formula is inadequate, as shown in Table 2, where results calculated by use of this formula are higher than those of the ketonic fraction. Thirdly, a fallacy, which is common to all formulae derived in this way, is introduced by the fact that certain ketosteroids, particularly those with an hydroxyl group at C_{11}, show a marked absorption peak in the violet (Fig. 1). In consequence, the presence of such compounds inevitably leads to false low values.

The addition of formalin prior to acid hydrolysis appears to obviate completely the necessity for the use of any correction formula. Estimations on 15 samples of urine, of varying ketosteroid content, have shown that the 17-ketosteroid excretion based upon the uncorrected green readings of the ketonic fraction and extracts of formalin treated urine do not differ significantly (Table 2). It is also seen that the 17-ketosteroid concentration calculated from the correction formula approximates the values derived from Girard fractionation and preliminary treatment of the urine with formalin.

DISCUSSION.

In clinical biochemistry rapid methods of estimation are desirable. However, in the estimation of urinary 17-ketosteroids, where a number of different compounds is estimated by the Zimmermann reaction in the presence of interfering chromogens, such methods may fail to give precise indication of adrenocortical activity.

TABLE 2.

17-ketosteroid values obtained by various methods of estimation.

Subject	17-K.S. (mg./l.) calculated from O.D. of:—				
	(a) Ketonic (green)	(b) Formalin (green)	Difference from Ketonic	(c) 1·38 G-B ÷0·74	Difference from Ketonic
Re.	4·2	3·9	−0·3	3·1	−1·1
Je.	4·0	3·5	−0·5	4·1	+0·1
Je.	16·2	16·3	+0·1	19·5	+3·3
Hu.	19·9	20·2	+0·3	20·4	+0·5
Br.	11·3	10·9	−0·4	12·2	+0·9
Ha.	1·5	1·0	−0·5	1·7	+0·2
Ke.	3·9	3·3	−0·6	3·2	−0·7
Ca.	5·5	5·8	+0·3	6·2	+0·7
Bu.	11·9	10·8	−1·1	12·5	+0·6
Gi.	35·4	35·4	0	37·0	+1·6
Sh.	9·6	9·7	+0·1	11·1	+1·5
Ba.	7·9	8·4	+0·5	8·2	+0·3
Fe.	5·7	6·1	+0·4	7·1	+1·4
Da.	15·5	15·5	0	15·7	+0·2
Sa.	6·2	5·5	−0·7	5·6	−0·6
Mean Difference			−0·16 ±0·12 (S.E.M.)		+0·59 ±0·28 (S.E.M.)

Acid hydrolysis is common to all rapid methods. It has been shown from Table 1 that at least one important steroid, dehydroisoandrosterone, is destroyed by this procedure. It has also been pointed out by Dingemanse, Huis in't Veld and Hartogh-Katz (1952) that certain other steroids are also altered under these conditions. The two 17-ketosteroids excreted in largest amounts in normal urines, androsterone and its isomer etiocholanolone, are not materially affected by acid hydrolysis. However, the extent of steroid degradation in urines from patients known to be suffering from secretory disorders of the adrenal cortex is always uncertain because of the destruction of such labile compounds during acid hydrolysis. Comparative chromatographic analysis of urine samples subjected to enzymic and acid hydrolysis shows the presence of a wider variety of 17-ketosteroids when the former method is used (Sheath, 1959).

A methanolic solution of potassium hydroxide has been employed as it is more stable than an ethanolic solution, and the Zimmermann colour produced in its presence obeys Beer's Law over the range required. This greater stability adds simplicity to the method described when compared with conventional techniques in which the ethanolic solution is employed.

The time and temperature selected for the Zimmermann reaction may greatly influence colour development. The time used in this method is a com-

promise between linearity of reaction and colour intensity. Similar findings have been reported by Wilson (1954).

The depth of colour of different ketosteroids produced in the Zimmermann reaction varies greatly. Urinary extracts contain a number of ketosteroids, as shown by Pond (1951), Dingemanse *et al.* (1952) and Savard (1953) each one of which may produce a different colour intensity from that produced by the pure 17-ketosteroid selected as standard.

In addition, a varying interference may be produced by non-steroidal chromogenic material. Both these factors almost certainly lead to errors of over- or under-estimation of 17-ketosteroids on occasions.

To attempt to eliminate this interference, the problem has been approached in three ways:

(a) The application of correction formulae to the Zimmermann reaction of crude extracts of urine. The final derivation of any such formula represents the mean of a large number of different urine extracts, which vary greatly in their non-ketonic chromogenic material. Therefore, serious errors may occur when this mean is applied to individual urines (Talbot *et al.*, 1942). The measurements of colour intensity between 510-530 mμ are based on the assumption that any given 17-ketosteroid has a maximum peak of absorption at this wavelength. Ketosteroids may be present which have a considerable degree of absorption in the violet. These may also give colours in the Zimmermann reaction which differ significantly from that of 17-ketosteroids. Thus, the use of any formula which attempts to correct for chromogenic material measured in the violet, may result in false values being introduced by contributions from true ketosteroids.

(b) Purification of the crude urine extract into its ketonic fraction (Girard fractionation). This procedure is time-consuming and impracticable for routine analyses.

(c) Addition of formalin to the urine prior to acid hydrolysis and extraction. Probably the best estimate of urinary 17-ketosteroids is achieved by the application of the Zimmermann reaction to the ketonic fraction and measuring the colour intensity at 520 mμ. The addition of formalin to the urine prior to hydrolysis in the determination of 17-ketosteroids appears to provide a distinct advance. A colourless urine extract is thus obtained, the colour intensity of which in the Zimmermann reaction does not differ significantly from that of the ketonic fraction. This technique therefore obviates the measurement of Zimmermann colour at two wavelengths, and the application of a correction formula. It is claimed that the formalin technique is not only more accurate, but that it is also time-saving.

Further, by comparing the colour intensity of a pure ketosteroid at 520 mμ with that of a formalin treated urine extract after the Zimmermann reaction the error introduced by 11-β-hydroxysteroids is obviated and a more reliable overall picture of the total 17-ketosteroids may be obtained.

Thus, the method recommended for routine use consists of the addition of 0·15 ml. formalin to 50 ml. urine, acid hydrolysis with 15 p.c. hydrochloric acid at 100° C. for 15 minutes, ether extraction and development of the Zimmermann colour for 45 minutes at 25° C.

The colour is then read against an appropriate blank at 520 mμ or with a green filter.

This technique is adequate to show gross disturbance in adrenal function (Table 3). However, it is not capable of giving an entirely true picture, par-

TABLE 3.

Urinary 17-ketosteroid excretion in health and disease, and the effect of ACTH stimulation.

Subject	Sex	Type	17-K.S. (mg./24 hr.)	
			Basal	Post ACTH
Ar.	Female	Normal	5·4	12·2
Bl.	Female	Normal	9·9	
Ed.	Female	Normal	8·1	
Fa.	Female	Normal	11·8	
Fe.	Female	Normal	11·3	23·5
Gr.	Female	Normal	6·8	23·4
McCa.	Female	Normal	8·0	
McCr.	Female	Normal	9·2	
Ne.	Female	Normal	8·6	
Pl	Female	Normal	13·6	
Si.	Female	Normal	5·6	24·4
Fa.	Male	Normal	15·9	
Fe.	Male	Normal	11·0	
Ge.	Male	Normal	11·6	
Kr.	Male	Normal	17·2	
La.	Male	Normal	10·7	
Hu.	Male	Normal	9·9	
Da.	Male	Addison	0·7	
Fa.	Female	Addison	1·1	0·4, 0·8, 0·7
Me.	Female	Addison	0·8	0·9
Ti.	Female	Addison	0·4	
Wh.	Female	Addison	0	0, 0·8, 0
Bu.	Female	Cushing	22·2	
Ma.	Female	Cushing	17·9	29·2, 45·0
Ro.	Female	Cushing	31·0	
Ty.	Male	Cushing	22·4	
De.	Female	Hirsutes	17·2	
Fr.	Female	Panhypo-pituitarism	0	3·9
Re.	Female	Post hypo-physectomy	0	0

ticularly if the excretion pattern rather than the quantity excreted is disturbed. Under such circumstances it may even be misleading.

Thus it would appear that the total ketosteroid excretion as determined by this technique should be supplemented in any doubtful case. When adrenal dysfunction is suspected, and the usual methods fail to indicate its presence,

this may be done by enzymic hydrolysis, isolation, identification and estimation of the individual steroids. Only by such techniques is it finally possible either to establish or eliminate disorders of adrenal steroid secretion or metabolism.

Acknowledgments. I wish to thank Dr. Ewen Downie, Dr. J. Bornstein and Dr. Bryan Hudson for their continued interest and encouragement in this work.

REFERENCES.

Antunes, Luis Nora (1956): J. clin. Endocrin. Metab., 16, p. 1125.
Cahen, R. L., and Salter, W. T. (1944): J. biol. Chem., 152, p. 489.
Callow, Nancy Helen, Callow, R. K., and Emmens, C. W. (1938): Biochem J., 32, p. 1312.
Dingemanse, Elizabeth, Huis in't Veld, Leonora G., and Hartogh-Katz, S. Lore (1952): J. clin. Endocrin. Metab., 12, p. 66.
Drekter, I. J., Heisler, A., Scism, G. R., Stern, S., Pearson, S., and McGavack, T. H. (1952): *Ibid.*, 12, p. 55.
Fraser, R. W., Forbes, Anne P., Albright, F., Sulkowitch, H., and Reifenstein, E. (1941): J. clin. Endocrin., 1, p. 234.
Gibson, J. G., and Evans, W. A. (1937): J. clin. Invest., 16, p. 301.
Gibson, J. G., and Evelyn, K. A. (1938): *Ibid.*, 17, p. 153.
Holtorff, A. F., and Koch, F. C. (1940): J. biol. Chem., 135, p. 377.
Krieger, Vera I. (1950): Med. J. Austral., 2, p. 678.
Landau, R. L. (1949): Am. J. clin. Path., 19, p. 424.
Langstroth, G. O., and Talbot, N. B. (1939): J. biol. Chem., 128, p. 759.
M.R.C. Committee on Clinical Endocrinology (1951): Lancet, 2, p. 585.
Pincus, G., and Pearlman, W. H. (1941): Endocrinology, 29, p. 413.
Pond, M. H. (1951): Lancet, 2, p. 906.
Savard, K. (1953): J. biol. Chem., 202, p. 457.
Sheath, June B. (1959): Austral. J. exp. Biol., 37, p. 147.
Talbot, N. B., Berman, R. A., and MacLachlan, E. A. (1942): J. biol. Chem., 143, p. 211.
Talbot, N. B., Butler, A. M., and MacLachlan, E. (1940): *Ibid.*, 132, p. 595.
Vestergaard, Per. (1951): Acta endocr., 8, p. 193.
Wilson, Hildegard (1954): Arch. Biochem. Biophys., 52, p. 217.
Zimmermann, W. (1935): Ztschr. physiol. Chem., 233, p. 257.
Zimmermann, W. (1936): *Ibid.*, 245, p. 47.

CHROMATOGRAPHY OF URINARY KETOSTEROIDS[1]

by JUNE B. SHEATH

(From the Diabetic and Metabolic Unit, Alfred Hospital, Melbourne).

(*Accepted for publication 11th November, 1958.*)

SUMMARY.

A paper chromatographic technique for the separation of urinary ketosteroids is described. Some of the problems of identification of the compounds recovered are discussed.

INTRODUCTION.

The determination of urinary ketosteroids by conventional techniques commonly fails to give an adequate estimate of androgenic function. Acid hydrolysis employed in these methods is known to destroy or modify certain steroids (Dingemanse, Huis in't Veld and Hartogh-Katz, 1952). Since different ketosteroids, when subjected to the Zimmermann reaction produce different absorption spectra, any determination of these substances based upon the assumption of a common absorption peak will lead to erroneous results. There are frequently marked differences in colour intensity produced by this reaction for equimolar concentrations of different steroids. Moreover, it is probable that the excretion pattern of steroids may be at least as significant as the total excreted.

Accordingly, it was decided to employ enzymatic hydrolysis and separation of the individual ketosteroids in an attempt to identify and estimate them individually.

This paper deals with a rapid paper chromatographic technique developed for the purpose of separating the compounds recoverable from urine. Chromatography of ketosteroids has been carried out either on adsorption columns (Dingemanse, Huis in't Veld and de Laat, 1946; Pond, 1951), paper chromatography with volatile solvents (Bush, 1952), or by the use of a volatile mobile phase and non-volatile stationary phase (Savard, 1953).

As paper chromatography was considered the more suitable of the two methods for this purpose, an attempt was made to design a system which would

---

[1] This paper was portion of the work submitted for the degree of Master of Science, Melbourne University.

separate steroids rapidly while not requiring incubator temperatures (Bush 1952).

A number of systems was investigated and the technique reported here has given consistently good separation of known compounds and those recovered from urine.

Reagents and Methods.

(1) Preparation of samples for chromatography. Suitable aliquots of urine are treated as follows:

(a) Hydrolysis with bacterial β-glucuronidase. To 50 ml. of urine is added 0·15M sodium acetate, and the pH adjusted to 6·5 with glacial acetic acid. Bacterial β-glucuronidase is added (350 units per ml. urine) and the mixture is left to stand for 72 hours at 32-37° C. The hydrolysed urine is then extracted with ether and the ether extract is washed with sodium hydroxide and distilled water.

(b) After extraction, acid hydrolysis of this urine is carried out. The hydrolysed urine is then extracted with ether according to the previously reported method (Sheath, 1959). The extracts are combined, dried and the ketonic fraction of the extract is separated by use of Girard's Reagent T according to the technique of Pincus and Pearlman (1941). The ketonic fraction is dissolved in ethanol and a suitable aliquot applied as a short line on the paper. A spot containing mixed known steroids is also applied. Application of the material to the paper is made so that the starting line is 6-8 cm. from the trough edge to control the initial rush of solvent onto the paper. The chromatogram is equilibrated and developed according to the technique described below.

(2) Chromatography paper.

Whatman No. 2 chromatography paper, after 24 hours' extraction with ethanol in a Soxhlet apparatus, has been found to be satisfactory.

(3) Solvent system.

2 vol. light petroleum ether (40-60° C. boiling range),
1 vol. distilled water,
1 vol. methanol, purified as previously described (Sheath, 1959),
Aqueous layer—stationary phase,
Petroleum ether layer—mobile phase.

(4) Tank.

A glass tank 8 in. square and 20 in. deep, fitted with a ground glass lid, is used. It is lined with filter paper, and wicks of filter paper are placed in beakers, within the tank. The tank is prepared for use as follows:

Mobile phase is run into the bottom of the tank to a depth of 2 cm. The beakers are filled with stationary phase; the lid is closed and the solvent system allowed to equilibrate for 48 hours at room temperature. Descending chromatograms have been found to give more satisfactory results than ascending chromatograms.

(5) Pre-equilibration and running of chromatograms.

The chromatograms are placed in the tank and allowed to equilibrate overnight. The mobile phase is then introduced into the trough through a suitably placed hole in the lid, and the chromatogram run for approximately 30 cm. This takes on the average about 3 hours.

(6) Development of colour.

The chromatograms are removed from the tank and dried in a stream of cold air. The ketosteroid spots are then developed with alkaline m-dinitrobenzene according to the

method of Savard (1953). Intensity of violet colour produced by ketosteroids with the Zimmermann reagents varies greatly. For example, 10 μg. of adrenosterone gives a greater intensity of colour than 25 μg. of androsterone.

Certain compounds recovered after hydrolysis of urine have been found to give an intense blue rather than a violet colour. Where these have been identified they have usually been 3-20-ketosteroids and not 17-ketosteroids.

RESULTS.

The technique as outlined has been applied to the separation of:

(a) Known steroid mixtures.

(b) Urinary extracts obtained by

(i) Acid hydrolysis.

(ii) β-glucuronidase hydrolysis.

(iii) Combined hydrolysis as described.

This method has satisfactorily separated all 17-ketosteroids available in the Alfred Hospital at the present time, and Fig. 1 shows a typical chromatogram of these compounds before and after heating with acid. It will readily be seen that the steroid pattern has been considerably distorted by acid treatment.

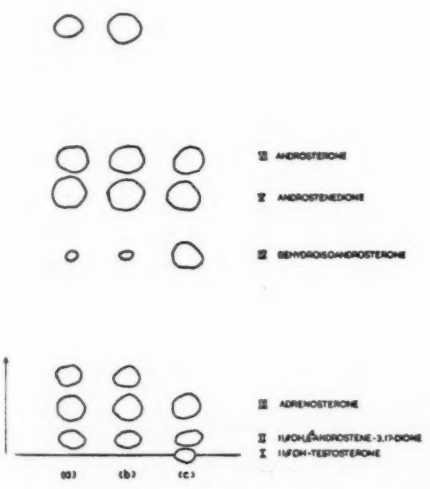


Fig. 1. The effect of acid hydrolysis on a mixture of pure steroids. (a) and (b) mixture after hydrolysis; (c) mixture before hydrolysis.

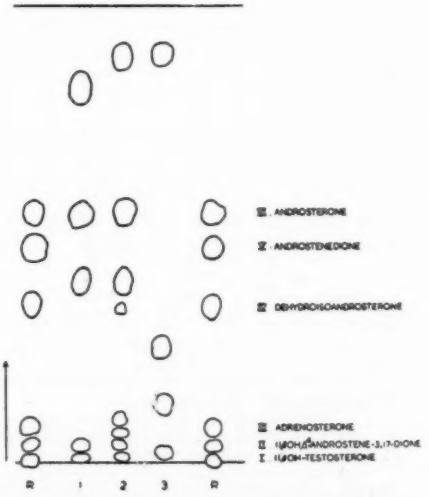


Fig. 2. Chromatogram of urinary steroids (subject A). Steroids obtained from urine after (1) acid hydrolysis; (2) enzymic hydrolysis; (3) acid hydrolysis after enzymic hydrolysis and extraction; (R) multiple reference standard.

Table 1 shows the mean Rf values obtained for the available steroids.

TABLE 1.

Average Rf values for pure C_{19} ketosteroids.

Steroid	Mean Rf value	Range	No. of experiments	Zimmermann colour
Androsterone	0·56	0·51-0·64	13	Violet
Androstenedione	0·48	0·43-0·57	12	Violet
Dehydroisoandrosterone	0·35	0·32-0·40	12	Violet
Adrenosterone	0·07	0·06-0·09	11	Violet
11, β, hydroxy-Δ^4-androstene, 3, 17-dione	0·02	0·01-0·03	11	Violet
11, β, hydroxytestosterone	0		11	Blue

TABLE 2.

Rf of Zimmermann reacting steroids recovered following hydrolysis of urines either by acid alone or by combined enzymic-acid methods.

Subject		A (B.H.)		B (A.E.)		C (M.C.)		D (S.J.)
Age Sex		35 male		30 female		27 female 7/12 pregnant		10 female
Hydrolysis		Acid	Combined	Acid	Combined	Acid	Combined	Combined
Rf of compounds recovered	1	—	0·90	0·86	0·88	0·92	0·91	—
	2	0·83	—	—	—	—	—	—
(a)	3	—	—	—	0·72	—	0·74	—
(b)	4	0·54	0·56	0·53	0·56	0·57	0·54	0·52
(c)	5	0·40	0·41	0·38	0·44	0·44	0·38	0·43
(d)	6	—	0·36	—	—	—	—	0·33
	7	—	—	—	—	0·31	—	—
	8	—	0·24	—	0·20	—	—	—
	9	—	0·13	—	—	—	—	—
	10	—	0·10	—	0·08	—	0·08	0·07
	11	0·05	0·06	0·05	—	0·04	0·05	—
(e)	12	0·03	0·03	0·02	0·02	0·02	0·02	0·02
	13	—	0·01	—	—	—	0·00	0·00

(a) This compound has sulphuric acid spectrum characteristics suggestive of either androstane-3-17-dione or etiocholane-3-17-dione.

(b) This compound has been identified by Rf value and sulphuric acid spectrum to be androstane-3-ol-17-one (androsterone).

(c) This compound has the characteristic sulphuric acid spectrum of etiocholane-3-ol-17-one.

(d) This compound has a sulphuric acid spectrum strongly suggestive of Δ^5-androstene-3-ol-17-one (dehydroisoandrosterone).

(e) This compound has a spectrum suggestive of hydrocortisone. The spot produced an intense blue colour with the Zimmermann reagents.

The other compounds have so far not been identified, either totally or partially.

The available reference compounds were run in all chromatograms.

Table 2 shows a comparison of the steroids obtained from samples of urine, when subjected to (a) acid hydrolysis, (b) combined enzymic and acid hydrolysis. The chromatogram obtained from subject A is shown in Fig. 2. This

chromatogram shows eight compounds after enzymic hydrolysis (2), and four compounds after acid hydrolysis following enzymic hydrolysis and extraction (3). In Table 2, ten compounds are shown, as two compounds in Fig. 2 had the same Rf values in both hydrolyses.

It is seen that certain of the steroids obtained by enzymic hydrolysis (particularly numbers 6, 8, 9 and 10) have been destroyed during acid hydrolysis, whereas other steroids are unaffected by acid hydrolysis (notably numbers 4, 5, 11 and 12). A fast running compound was obtained from the three adult urines subjected to combined hydrolysis. In the case of subject A following acid hydrolysis a fast running compound appeared which was not detected after combined hydrolysis.

In order to determine whether real destruction occurs during acid hydrolysis, the pure ketosteroids androsterone, androstenedione and dehydroisoandrosterone were chromatographed before and after acid hydrolysis. The mean results are presented in Table 3.

TABLE 3.

Rf values of pure ketosteroids chromatographed before and after acid hydrolysis.

Steroid	Treatment	
	Nil	Acid
Androsterone	0·57	0·56
Androstenedione	0·46	0·46
Dehydroisoandrosterone	0·36	0·36 (Trace)

It is thus seen that when present in low concentrations there is a total destruction of dehydroisoandrosterone, whereas when it is present in higher concentrations, as in Fig. 1, some is recovered as such, and a portion as a faster moving compound.

DISCUSSION.

The method described provides a rapid means of separating certain ketosteroids with relative ease and simplicity. It is at present being applied to the study of the excretion patterns in normal controls and to the investigation of patients showing evidence of abnormal adrenal function.

At times some difficulty has been encountered, because some very slowly moving compounds have tended to tail into each other. Under such circumstances these have been eluted and run for a longer period, the chromatogram being permitted to overflow, as in the technique of Savard (1953). A further problem is that both C_{19} ketosteroids and C_{21} ketosteroids are extracted by this

technique and hence it is desirable to separate them prior to paper chromatography. This is readily accomplished by adsorption chromatography on Florosil according to the method of Nelson and Samuels (1952). The identification of the individual steroids presents considerable problems. The best method available at present is infra-red spectrophotometry, but this equipment is not generally available and fairly large quantities of material are required.

When pure steroids are available, reasonable identification may be obtained by showing that the pure compound and the unknown have similar Rf values in several chromatographic systems. If these are not obtainable then other methods must be used. Sulphuric acid spectrophotometry (Zaffaroni, 1950; Bernstein and Lenhard, 1953) has been investigated and up to the present time, as shown in Table 2, five compounds have been identified by this method. In three cases the pure compound was available, but in the other two (etiocholane-3-ol-17-one and androstane-3-17-dione) identification was made by this technique. This procedure is being extensively investigated at the present time and offers considerable promise. Other techniques available to the ordinary laboratory are the use of 2-4-dinitrophenylhydrazine spectra (Gornall and Macdonald, 1953), alkaline fluorescence (Bush, 1952), sulphuric acid fluorescence (Sweat, 1954), and a number of other colour reactions. These are at present being investigated on compounds recovered from urine and the results will be reported later.

Acknowledgments. Part of the expenses of this study were defrayed from a generous grant given by the Upjohn Company, Kalamazoo, Michigan, U.S.A. The kindness of Dr. F. T. Johnson who provided many of the reference steroids for this investigation is gratefully acknowledged.

My thanks are due to Dr. Ewen Downie, Dr. J. Bornstein and Dr. Bryan Hudson for their continued interest and encouragement in this work.

REFERENCES.

Bernstein, S., and Lenhard, R. (1953): J. org. Chem., 18, p. 1146.
Bush, I. E. (1952): Biochem. J., 50, p. 370.
Dingemanse, Elizabeth, Huis in't Veld, Leonora G., and de Laat, B. M. (1946): J. clin. Endocr., 6, p. 535.
Dingemanse, Elizabeth, Huis in't Veld, Leonora G., and Hartogh-Katz, S. Lore (1952): J. clin. Endocr. and Metab., 12, p. 66.
Gornall, A. G., and Macdonald, M. Pamela (1953): J. biol. Chem., 201, p. 279.
Nelson, D. H., and Samuels, L. T. (1952): J. clin. Endocr. and Metab., 12, p. 519.
Pincus, G., and Pearlman, W. H. (1941): Endocrinology, 29, p. 413.
Pond, M. H. (1951): Lancet, 2, p. 906.
Savard, K. (1953): J. biol. Chem., 202, p. 457.
Sheath, June B. (1959): Austral. J. exp. Biol., 37, p. 133.
Sweat, M. L. (1954): Anal. Chem., 26, p. 1964.
Zaffaroni, A. (1950): J. Am. Chem. Soc., 72, p. 3828.

ELECTRON MICROSCOPY OF THE REPTILIAN RENAL GLOMERULUS

by R. K. F. PAK POY

(From the Department of Pathology, University of Adelaide).

(*Accepted for publication 10th November, 1958.*)

SUMMARY.

The metanephric reptilian renal glomerulus frequently contains a Central Cell Mass. The mass is not as large as in the avian glomerulus, often being only slightly greater in amount than the intercapillary tissue seen in other vertebrate glomeruli. The capillary wall is very similar to that seen in the amphibian and piscine renal glomerulus, possessing a peri-capillary space which may contain tissue. The visceral epithelial layer is relatively simple.

INTRODUCTION.

Electron microscopical studies of the renal glomerulus of different classes of vertebrates have been reported by numerous workers. The structure of the higher mammalian glomerulus has been reported by Pease and Baker (1950), Dalton (1951), Oberling *et al.* (1951), Hall (1953, 1954), Reid (1954), Mueller *et al.* (1955), Pease (1955), Yamada (1955), Rinehart (1955), Sakaguchi (1955), Bergstrand (1957), Farquhar *et al.* (1957), and Pak Poy (1958a). The marsupial and avian glomeruli have been studied by Pak Poy (1957a) and Pak Poy and Robertson (1957). The structure of the amphibian renal glomerulus has been reported by Fawcett (1954), Bargmann *et al.* (1955) and Pak Poy (1957b), and of the fresh water piscine renal glomerulus by Pak Poy (1958a). The present paper describes the structure of the metanephric (Young, 1950) renal glomerulus of the lizard (*Trachysaurus rugosa*). The presence of a large mass of tissue in the centre of the capillary tuft of the reptilian glomerulus has been described in conventional light microscopical studies. Vilter (1935) described the mass of tissue in the glomeruli of the horned toad as being morphologically similar to that of the Central Cell Mass of the avian renal glomerulus except that the nuclei are not as compactly arranged, and that it was as constant a feature as in the avian glomerulus. Smith (1943) described the nature of the mass as inert connective tissue, and considered the reptilian glomerulus to be a degenerate structure with the capillary tuft consisting of only four capillaries (Smith, 1951). Edwards and Schnitter (1933) described the glomerulus of the horned toad as being well vascularised, but in their Fig. (46) the glomerulus of the horned toad shows a well marked central core of tissue.

Materials and Methods.

Animals were anaesthetised by intra-peritoneal nembutal supplemented by inhaled ether. The kidneys were exposed and fixed with 2 p.c. osmium tetroxide in acetate-veronal buffer (0·14M and pH 7·4) for ten minutes at 4° C. Dehydration, embedding and sectioning were carried out in the routine manner and the sections were examined in a Philips electron microscope type EM100.

RESULTS.

Central Cell Mass.

In numerous glomeruli, although not as constantly present as in the avian glomerulus, there is a mass of tissue expanding into the centre of the glomerulus, and because of its marked morphological similarity to that in the avian glomerulus it has been decided to call this mass of tissue the Central Cell Mass. In the reptilian glomerulus the Central Cell Mass varies considerably in size, ranging from a small mass comparable to the amount of intercapillary tissue seen in the mammalian glomerulus to a mass almost as large as the Central Cell Mass of the avian glomerulus (Fig. 1). The Central Cell Mass of the reptilian glomerulus is composed of separate cells with intercellular material between them, but they are not as compactly arranged as in the avian glomerulus. The surface of the mass is very irregular and is separated from blood by the endothelial lining of the capillaries, and from Bowman's space by the dense layer of the capillary basement membrane, which runs around the surface of the Central Cell Mass from capillary to capillary. The tissue of the Central Cell Mass does not have the marked increase in density in comparison with the endothelium and epithelium as in the avian glomerulus.

The cells of the Central Cell Mass are large with irregular shaped nuclei, which may contain a nucleolus. The chromatin network is evenly distributed and is surrounded by a double nuclear membrane. The cytoplasm is irregular in shape and there are many projections of cytoplasm present without nuclei, but they do not form as complex a pattern as in the avian glomerulus. In the cytoplasm there are mitochondria, ribonucleoprotein (R.N.P.) granules and endoplasmic reticulum. Except for its position there are no features to distinguish the cell of the Central Cell Mass from the endothelial cell.

The intercellular space of the Central Cell Mass is filled with a mottled material, which is continuous with the mottled material in the inner less dense layer of the capillary wall when it is present. The thin layer of dense material commonly seen in the intercellular material of the Central Cell Mass of the avian glomerulus is not present in the reptilian glomerulus. At the junction of the capillary wall with the Central Cell Mass, the thin sub-epithelial dense layer, which has been designated the capillary basement membrane, continues around the surface of the mass exposed to Bowman's space. Mottled intercellular material generally separates this layer from the cells of the Central

Cell Mass, just as it generally separates the endothelium from the capillary basement membrane. The endothelium of the capillaries is separated from the cells of the Central Cell Mass usually by mottled intercellular material, but occasionally by dense homogeneous material.

Glomerular loops.

There are relatively few capillary loops spread over the surface of the Central Cell Mass, and some of the lumina are extremely small and in close proximity to the surface of the Central Cell Mass. Basically, the morphology of the endothelium is the same as in all other glomeruli, being composed of separate cells which give rise to thin attenuated layers of cytoplasm lining the greater part of the capillary lumen. However, it differs from the endothelium of mammalian and avian glomeruli in that the bodies of the endothelial cells are scattered haphazardly around the capillary, and that "pores" in the thin attenuated layer are extremely scarce.

Visceral epthelium.

The morphology of the visceral epithelial layer is essentially the same as in the other vertebrate glomeruli, being composed of cells lying free in Bowman's space and giving rise to trabeculae and pedicels which rest on the outer surface of the capillary wall. The cells are more numerous than the endothelial cells, and are smaller than the visceral epthelial cells of the glomeruli of higher vertebrates. Generally, the bodies of the cells are situated quite close to the capillary wall, and frequently they are in contact with the capillary wall without much distortion of the cell outline (Fig. 3), which is much more rounded than the very irregular shapes seen in higher vertebrate glomeruli. The trabeculae are few, relatively simple and do not form long sinuous processes of cytoplasm. Likewise, the pedicels form a relatively simple system, resembling that seen in the amphibian glomerulus. They are small oval or rounded masses of cytoplasm which vary considerably in size, and in tangential section the interdigitating pattern is relatively simple. As well as covering the external surfaces of the capillaries, the pedicels also cover the surface of the Central Cell Mass exposed to Bowman's space. In the cytoplasm of the cell bodies, trabeculae and pedicels, there are the usual organelles – mitochondria, ribonucleoprotein granules and endoplasmic reticulum – and occasionally a moderately large particle composed of dense homogeneous material, resembling somewhat the protein droplets described in the tubular cells (Rhodin, 1954). The limiting cytoplasmic membrane is clearly defined, with a slight increase in density at the junction with the capillary wall.

The nucleus of the epithelial cell is generally round or ovoid, with a smooth surface limited by a double membrane. The chromatin network is evenly distributed, and occasionally a nucleolus is present.

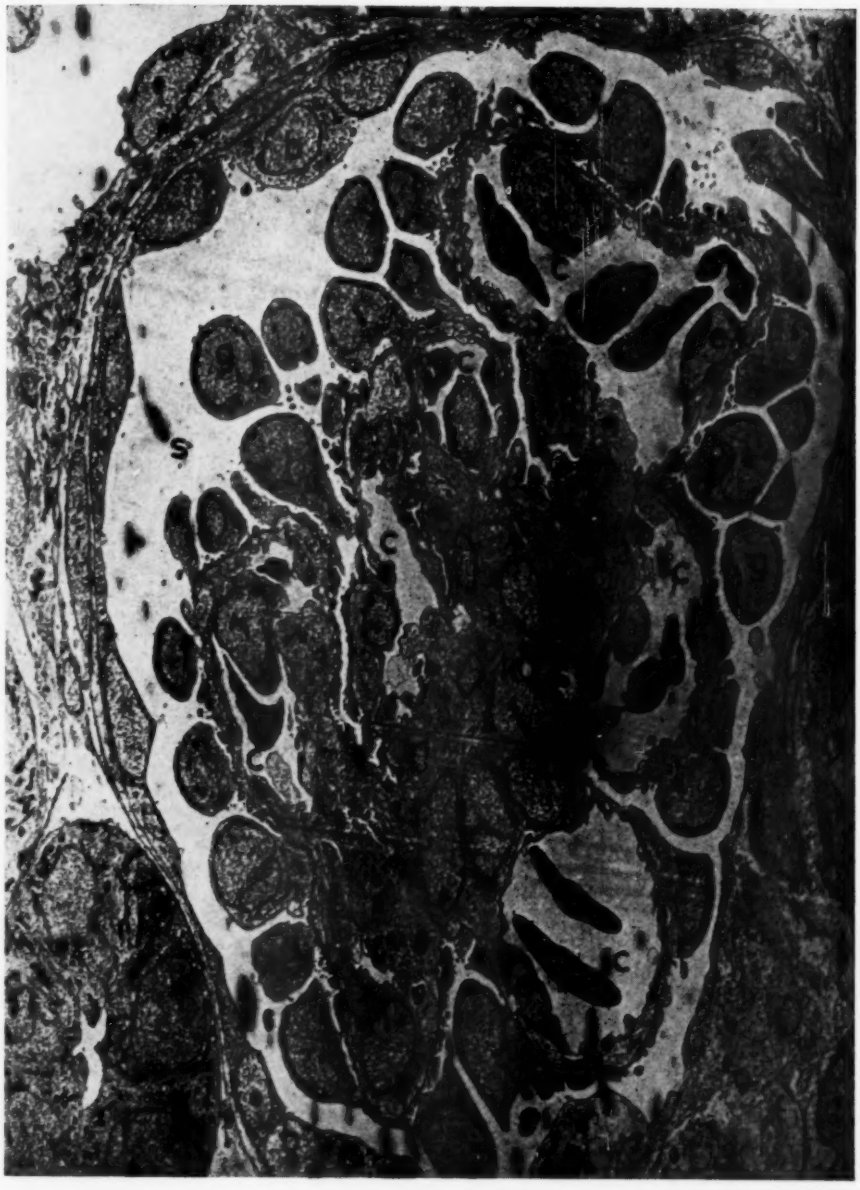
Fig. 1. This low power electron micrograph shows the Central Cell Mass (k) with a small number of capillary loops (c) scattered over its surface. The endothelial cells (e) are scattered haphazardly around the capillary loops. The visceral epithelial cells (g) form a relatively simple layer in Bowman's space (s) and cuboidal cells (b) are present in the parietal epithelial layer.

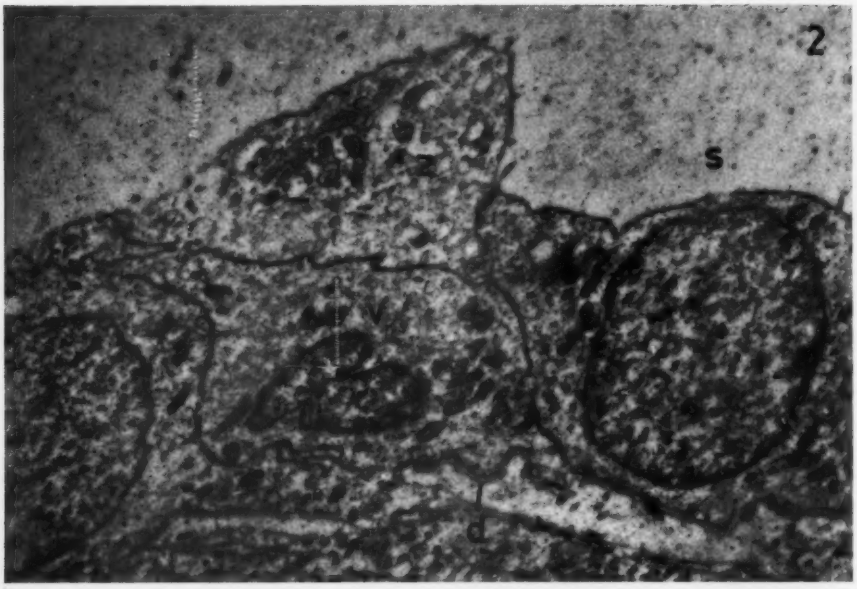
Fig. 2. This electron micrograph of Bowman's capsule shows the thin dense basement membrane at (d) and Bowman's space at (s). In the parietal epithelial layer the cytoplasm of cell (z) overlies the cell (v). × 20,000.

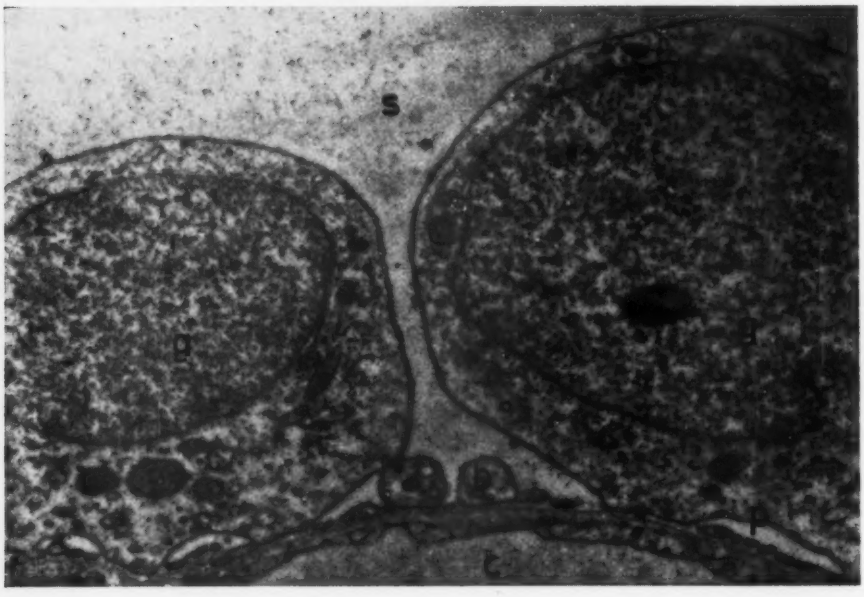
Fig. 3. Bowman's space is at (s) and a capillary lumen is at (c). The epithelial cells (g) are reasonably round with a regular smooth surface, and are situated close to the capillary wall, with which they occasionally come into contact without much distortion of the cell outline. A "blob-like" pedicel is at (b) and a small chain of "droplet-like" pedicels is at (p). This visceral layer is simple in comparison with the mammalian glomerulus. × 20,000.

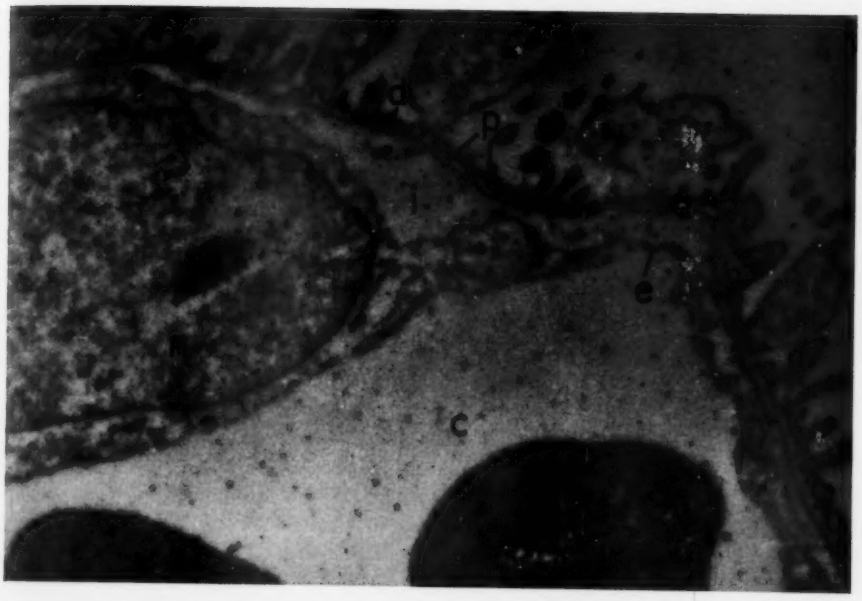
Fig. 4. The capillary lumen (c) is lined by an endothelial cell (n) giving rise to the attenuated layer of cytoplasm (e). The capillary wall basement membrane is a thin dense layer (d) which is separated by a thin less dense layer (o) from the epithelial pedicels (p). The enlarged inner less dense layer (i) has been termed the peri-capillary space. × 20,000.

Fig. 5. This shows a mass of cytoplasm (k) in the peri-capillary space (h) which is separated from the epithelium by the dense basement membrane (d) and from the capillary lumen (c) by the endothelial layer (e). × 20,000.


S
C
C
C


2
S
V
d


S
g
t


d
P
l
e
c

c

Capillary wall.

The wall of the peripheral part of the capillary is generally composed of the attenuated endothelium, the epithelial pedicels and the structures between these two layers. In this intercellular space between the two cytoplasmic layers, there is the capillary loop basement membrane, which is a thin dense layer measuring approximately 300Å, and is almost always sub-epithelial in position, being separated from the epithelial pedicels by a thin less dense layer (Fig. 4). This outer less dense layer is a reasonably constant structure and does not vary much in thickness, measuring approximately 300-500Å. However, the space between the endothelium and the capillary loop basement membrane varies considerably in width and frequently contains a mottled material, which as stated earlier is continuous with the intercellular material of the Central Cell Mass. When this space is very wide it is termed the peri-capillary space, as it corresponds to the potential space described by Jones (1957) and earlier by McCallum (1934).

Peri-capillary tissue.

As in the mammalian, amphibian and fresh water piscine glomerulus, projections of cytoplasm are quite frequently present in the peri-capillary space, even at the periphery of the glomerulus (Fig. 5). The dense capillary basement membrane layer separates the peri-capillary tissue from the epithelial pedicels in the same way as it separates the tissue of the Central Cell Mass from the epithelial pedicels in Bowman's space.

Bowman's capsule.

The parietal epithelium lining Bowman's capsule is generally composed of a single layer of flattened separate cells with flattened nuclei. Frequently, however, the cells are cuboidal or pyramidal with ovoid nuclei, and very occasionally the parietal epithelium is two layers thick with the cytoplasm of one cell overlying the cytoplasm of the adjacent cell. In the cytoplasm adjacent to the basement membrane, there are on some occasions filaments of dense material running at an acute angle to the cell membrane with which they occasionally come into contact, obscuring its definity. The capsular basement membrane is a very thin layer of dense material measuring approximately 300Å and is usually separated from the epithelium by a thin, less dense layer.

Hilum.

At the hilum the capsular basement membrane is continuous with the dense sub-epithelial basement membrane of the capillary, and this continuous layer bears the same relationship to the adventitial coat of the arteriole as it does to the Central Cell Mass.

DISCUSSION.

There are too many cells in the Central Cell Mass, and too few capillary loops for the cells of the Central Cell Mass to be endothelial cells belonging to capillaries in other planes, and so the Central Cell Mass is certainly intercapillary tissue as in the avian glomerulus. The presence of very small capillary lumina in close proximity to the surface of the Central Cell Mass gives the glomerulus an appearance resembling the developing mammalian glomerulus (Hall, 1956; Kurtz, 1958). Furthermore, the relatively simple visceral epithelial layer, the frequent presence of cuboidal cells in the parietal epithelium, the thin capsular basement membrane and the thin sub-epithelial basement membrane of the capillaries are all features found in the developing human and rat's glomerulus. It is suggested that the reptilian glomerulus is a poorly developed structure, and that the Central Cell Mass is the remnant of the mesenchymal anlage from which the glomerulus develops.

The lesser complexity of the visceral epithelial layer and its close resemblance to the visceral epithelial layer in the amphibian and piscine glomerulus brings to light an interesting point in the comparative morphology of the vertebrate glomerulus. In mammals and birds the blood pressure is high (120-200 mm. Hg.) and the visceral epithelium is complex. However, in the lower vertebrates the blood pressure is low (40 mm. Hg.) (Woodbury and Hamilton, 1937) and the visceral epithelium is comparatively simple. Pappenheimer *et al.* (1951) have shown in their physical study of capillary permeability that the filtration constant in the mammalian renal glomerular capillary is greater than in the amphibian glomerular capillary and also than in the mammalian capillaries in other parts of the body. In this comparative study it has been seen that the complexity of pedicellar system in the mammalian glomerulus is more complicated than in the amphibian glomerulus and is also more complicated than cell surface invaginations in other areas of fluid transfer across capillary walls in other parts of the mammalian body. Further work is necessary to establish if there is any relationship between visceral epithelial complexity and blood pressure and filtration constant.

The extreme paucity of pores in the endothelium may be related to the short fixation of the tissue and suggests that these pores may possibly be artefactual due to over fixation.

Acknowledgments. The author would like to thank Dr. S. G. Tomlin for granting access to the electron microscope and Professor J. S. Robertson for continued interest and criticism in this study.

REFERENCES.

Bargmann, W., Knoop, A., and Schiebler, Th. H. (1955): Z. Zellforsch., 42, p. 386.
Bergstrand, A. (1957): Lab. Invest., 6, p. 191.
Dalton, A. J. (1951): J. Nat. Cancer Inst., 11, p. 1163.

Edwards, J. G., and Schnitter, C. (1933): Amer. J. Anat., 53, p. 55.
Farquhar, M. G., Vernier, R. L., and Good, R. A. (1957): Amer. J. Path., 33, p. 791.
Fawcett, D. W. (1954): Anat. Rec., 120, p. 733.
Hall, B. V. (1953): Proc. 5th Ann. Conf. Nephrotic Syndrome, Philadelphia, Josiah Macy, Jr., Foundation.
Hall, B. V. (1954): Proc. 6th Ann. Conf. Nephrotic Syndrome, Cleveland.
Hall, B. V., and Roth, L. E. (1956): Electron Microscopy Proc. Stockholm Conf.
Jones, D. B. (1957): Amer. J. Path., 33, p. 313.
Kurtz, S. M. (1958): Exp. Cell Res., 13, p. 417.
McCallum, W. G. (1934): Bull. Johns Hopkins Hosp., 55, p. 416.
Mueller, C. B., Mason, A. D., Jr., and Stout, D. G. (1955): Amer. J. Med., 18, p. 267.
Oberling, C., Gautier, A., and Bernhard, W. (1951): Presse Méd., 59, p. 938.
Pak Poy, R. K. F. (1957a): Austral. J. exp. Biol., 35, p. 437.
Pak Poy, R. K. F. (1957b): *Ibid.*, 35, p. 583.
Pak Poy, R. K. F. (1958a): *Ibid.*, 36, p. 191.
Pak Poy, R. K. F. (1958b): Amer. J. Path., 34, p. 885.
Pak Poy, R. K. F., and Robertson, J. S. (1957): J. biophys. biochem. Cytol., 3, p. 183.
Pappenheimer, J. R., Renkin, E. M., and Bonero, L. M. (1951): Amer. J. Physiol., 167, p. 13.
Pease, D. C., and Baker, R. F. (1950): Amer. J. Anat., 87, p. 349.
Pease, D. C. (1955): J. Histochem. Cytochem., 3, p. 295.
Reid, R. T. W. (1954): Austral. J. exp. Biol., 32, p. 235.
Rhodin, J. (1954): Correlation of Ultrastructural Organisation and Function in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of Mouse Kidney.
Rinehart, J. F. (1955): Arch. Path., 59, p. 439.
Sakaguchi, H. (1955): Keio. J. Med., 4, p. 103.
Smith, H. W. (1943): Lectures on the Kidney, Univ. of Kansas, Kansas.
Smith, H. W. (1951): The Kidney, Oxford Univ. Press, New York.
Vilter, R. W. (1935): Anat. Rec., 63, p. 371.
Woodbury, R. A., and Hamilton, N. F. (1937): Amer. J. Physiol., 71, p. 209.
Yamada, E. (1955): J. biophys. biochem. Cytol., 1, p. 551.
Young, J. Z. (1950): Life of Vertebrates, Oxford Univ. Press.

FURTHER STUDIES OF AN AVIRULENT MUTANT OF THE INFLUENZA A STRAIN WS—

by PATRICIA E. LIND[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication 7th November, 1958.*)

SUMMARY.

Investigation of the behaviour of aWS—, a non-mouse-pathogenic mutant of the influenza strain WS—, on intranasal inoculation into mice has shown that, although it is adsorbed to mouse lung at the same rate as the mouse-pathogenic WS— strain, it fails to multiply in mouse lung tissue. Inoculation of large doses of aWS— does not give rise to "toxic" congestive lesions in the mouse lung.

Intranasal inoculation of aWS— into mice is followed by the production of neutralizing antibodies in the serum which protect the majority of mice against the lethal effects of subsequent challenge with WS—. Although antibody to the soluble complement fixing antigen of type A influenza is produced in mice in response to intranasal inoculation of sublethal doses of WS—, it is not produced in response to inoculation of aWS—.

The serological response of ferrets to intranasal inoculation of aWS— is essentially similar to that observed in mice.

Recombination experiments between aWS— and a fully mouse-pathogenic revertant strain of WS— to which it gave rise were carried out in de-embryonated eggs. A small number of recombinant strains of intermediate levels of mouse virulence were obtained by limit dilution titration of the harvest from the doubly infected eggs.

INTRODUCTION.

The characteristics of aWS–, a non-mouse-pathogenic mutant of the influenza virus strain WS–, and its reversion to mouse virulence under certain conditions of passage in the chick embryo have been described in a recent paper (Lind and Burnet, 1958). With the object of reaching a clearer understanding of the difference between aWS– and WS–, investigations were made of the behaviour of aWS– on intranasal inoculation into mice and of the results of recombination between aWS– and one of the mouse pathogenic revertant strains to which it gave rise. The findings are reported in this paper.

MATERIALS AND METHODS.

The characteristics of the two virus strains, aWS— and WS—, used in this study together with many relevant techniques have already been described in the first paper of this

[1] Aided by a grant from the National Health and Medical Research Council, Canberra.

series (Lind and Burnet, 1958). Only the method of assessing mouse lung pathogenicity and methods not previously described are mentioned here.

Definition of virus dose. The haemagglutinin (HA) titre of an influenza virus preparation is determined by estimating the highest dilution which, when added in a volume of 0·25 ml. to 0·25 ml. of a 1 p.c. suspension of fowl red blood cells, will produce a standard degree of partial agglutination of the fowl cells. Virus, at the dilution of the end-point, is considered to be diluted to the level of 1 agglutinating dose (1 AD). The terms 5 AD and 10 AD represent virus in 5 and 10 times this concentration respectively.

Titration of the anti-haemagglutinin (anti-HA) content of sera. To destroy non-specific inhibitors of haemagglutination the sera were first treated with the receptor destroying enzyme of *V. cholerae* (RDE). Mixtures consisting of 0·1 ml. serum, 0·25 ml. RDE (titre 1200) and 0·1 ml. calcium acetate buffer saline pH 6·2 (Stone, 1949) were incubated at 37° C. for 2 hours; 0·05 ml. of 20 p.c. sodium citrate was then added to inhibit the RDE. The mixture, containing virus diluted 1/5, was then heated at 56° C. for 30 minutes before titration.

Serial two-fold dilutions of the treated sera were prepared in 0·25 ml. volumes of saline and, to each, 0·25 ml. of virus diluted to 5 AD was added. This was followed by the addition of 0·25 ml. of a 1 p.c. fowl red cell suspension. The reciprocal of the serum dilution in which the standard degree of partial agglutination occurred after 45 minutes at room temperature was taken as the end-point.

Titration of the complement-fixing (CF) antibody. Titrations of antibody to the soluble influenza type A complement-fixing antigen were carried out according to Donnelley's method (1951). The purified antigen was prepared by differential ultra-centrifugation from chick embryo lungs infected with the standard type A strain, PR8 (Ada, Donnelley and Pye, 1952). Titres were expressed as the reciprocal of the dilution of serum which, in the presence of a standard dose of antigen, showed the 50 p.c. haemolysis end-point.

Determination of mouse lung pathogenicity (MLP). Virus fluids were diluted to 1 AD in saline containing 10 p.c. inactivated horse serum. 0·05 ml. amounts of this dilution were inoculated intranasally into groups of 4 - 8, 4-week-old mice under light ether-chloroform anaesthesia. The mice were observed for 7 days and all deaths recorded. The surviving mice were then killed and their lungs examined for areas of consolidation. Scores were made as follows: death on the fifth day or earlier scored 5, on the sixth day 4·5 and on the seventh day 4. Lesions in the surviving mice were scored 0 - 3·9 according to the degree of consolidation.

Method of obtaining bronchial washings. Bronchial washings were obtained from mouse lungs by the method of Fazekas de St. Groth (1948). Briefly, the mice were killed by over-anaesthetization and the trachea-lung complex excised. The upper end of the trachea was fastened to a cannula inserted in a rubber stopper. The stopper was then placed in a short test tube with a side arm by means of which the pressure in the test tube could be raised or lowered. Small volumes of saline, etc., placed in the cannula could be drawn into the lungs by lowering the pressure in the test tube and aspirated by increasing it.

In ovo *neutralization of virus by immune serum.* Serial dilutions of virus were prepared in ten-fold steps in chilled saline containing 10 p.c. inactivated horse serum. The serum under test was diluted 1/18 and 0·45 ml. amounts measured into a series of tubes. Appropriate virus dilutions, in 0·05 ml. amounts, were added to each of the serum tubes to give the final range of virus dilutions required and a final serum dilution of 1/20. After 15 minutes at room temperature each serum-virus mixture was inoculated allantoically into 5, 11-day chick embryos which were incubated at 35° C. for 3 days, then tested for the presence of virus in the allantoic fluid. The 50 p.c. infectivity end-points (EID_{50}) were calculated by the method of Reed and Muench (1938) and the results compared with those of a titration of virus diluted in horse serum saline alone.

Preparation of first cycle fluids in the de-embryonated egg (DEE). The method of Burnet and Lind (1954a) with minor modifications introduced by Lind and Burnet (1957) was used.

EXPERIMENTAL.

Multiplication of aWS– in mouse lung.

The question of whether the inability of aWS– to produce lesions in mouse lung was due to its failure to multiply in the lung tissue, or whether it multiplied but failed to give rise to lesions was investigated in the following experiment. Fifth limit dilution passage aWS– was diluted to 10 AD and 1 AD and 0·05 ml. of each dilution inoculated intranasally into 6 mice. Three days later 2 mice in each group were killed and bronchial washings were taken from the excised lungs. The washings were pooled in their respective groups and inoculated allantoically into chick embryos at a dilution of 10^{-2}. Neither pool caused infection. The remaining mice in each group were kept as controls and, when killed 7 days after infection, were found to be free of pulmonary lesions.

In the second experiment 6 mice were inoculated intranasally with 1 AD of aWS– and 2 killed on each of the 1st, 2nd and 3rd days after infection. Bronchial washings were taken as before. Those from the 1st day failed to infect chick embryos even when inoculated undiluted. Those taken on the 2nd day gave a titre of $10^{0.5}$ and the third day washings a titre of $10^{0.0}$. Two virus fluids isolated from the 2nd day bronchial washings were tested in mice at 1 AD dilution; both gave definite but minimal MLP scores of 0·1. These results clearly indicated that multiplication of aWS– in mouse lung is minimal.

Adsorption of aWS– to mouse bronchial epithelium.

Following these results, investigations were carried out to see if the poor multiplication of aWS– in mouse lung could be attributed to an initial failure to be adsorbed to the cells of the bronchial epithelium. Excised lungs from normal mice were attached to cannulae inserted into the trachea and mounted

TABLE 1.

HA titres of samples from excised mouse lungs inoculated with WS– and aWS–

Time of removal of sample (minutes)	Inoculum			
	100 AD WS–		100 AD aWS–	
	HA titre of sample		HA titre of sample	
0	100	100	100	100
5	8	12	4	2
10	2	< 2	< 2	< 2
15	< 2	< 2	< 2	< 2
30	< 2	< 2	< 2	< 2

as described under Methods. After washing out the lungs twice with saline, two sets were inoculated with 0·5 ml. of aWS– diluted to 100 AD and two with the same amount of WS–. At intervals the fluid was expressed from the lungs and replaced by 0·5 ml. of saline. The HA titres of the samples are shown in Table 1 and indicate that both aWS– and WS– are adsorbed to mouse lung at a similar rate.

Immunization of mice with active aWS–.

Several experiments were carried out on the effect of intranasal inoculation of mice with aWS– in protecting against subsequent intranasal inoculation with WS–.

In the first of these, groups of 12 mice were inoculated intranasally with 1 AD $\times 10^{-1}$, 1 AD and 10 AD of aWS–. Fourteen days later each group of 12 was divided into 3 sub-groups of 4 mice each and these were challenged by intranasal inoculation of 1 AD $\times 10^{-1}$, 1 AD and 10 AD of WS– respectively. Controls consisted of hitherto uninoculated mice of the same age which were given 1 AD $\times 10^{-3}$, 1 AD $\times 10^{-2}$ and 1 AD $\times 10^{-1}$ doses of WS–. The results, which are given in Table 2, reveal a substantial immunity to WS– infection among the majority of mice which had been pre-inoculated with aWS– and this immunity was proportional to the dose given.

TABLE 2.

Average MLP scores of groups of mice challenged with WS– 14 days after intranasal immunization with aWS–.

Challenge dose of WS–	Immunizing dose of aWS–			
	nil	1 AD $\times 10^{-1}$	1 AD	10 AD
1 AD $\times 10^{-3}$	2·2			
1 AD $\times 10^{-2}$	3·5			
1 AD $\times 10^{-1}$	4·3	0·2	<0·1	0·8+
1 AD		2·5	1·3+	<0·1
10 AD		4·3	1·9+	0·9

+Average value raised by the presence of one non-immune mouse in the group.

Two further experiments were carried out in which mice were given two immunizing doses of 1 AD aWS– a fortnight apart and challenged with varying amounts of WS– after the second dose. In both these experiments the mice were bled from the tail before each immunizing dose and immediately before challenge. In the first, the sera were tested for their WS– anti-HA content and, in some cases, for antibody to type A influenza virus soluble complement fixing antigen (CF antibody). The results, shown in Table 3, indicate a rise in anti-HA titres of the sera of all mice following immunization, but no absolute correlation between the level of serum antibody and resistance to WS– challenge. In the two cases in which there was sufficient serum for CF antibody titrations

no CF antibody could be demonstrated at the lowest dilution tested (1/5). In consequence of this finding, all sera from the second experiment were tested for their CF antibody levels. The results obtained in this experiment were essentially the same as those shown in Table 3. None of the mice showed any rise in the CF antibody titre of the serum.

TABLE 3.

Serological and immune response of mice immunized with aWS−.

Immunized mice	Anti-HA titres of sera			CF titres of sera			Lesion scores after challenge with WS−			
							Dose of WS− (AD)			
	I	II	III	I	II	III	0·01	0·1	1·0	10
1	<5	60	60						0·5	
2	<5	7	30						0·0	
3	<5	10	30						0·7	
4	<5	15	50						0·6	
5	<5	<5	8	<5	<5	<5				0·4
6	<5	<5	20	<5	<5	<5				5·0
7	<5	15	40							0·5
8	<5	<5	10							0·2
9	<5	<5	35							4·0
Non-immunized mice										
1	<5						2·7			
2	<5						4·0			
3	<5						2·5			
4	<5						2·3			
5	<5							3·0		
6	<5							1·5		
7	<5							4·0		
8	<5							3·6		
9	<5								5·0	
10	<5								5·0	
11	<5								5·0	
12	<5								5·0	

All the sera were stored at −12°C after collection and titrated for anti-HA at the same time, after treatment with RDE.

In all experiments a small proportion of mice failed to develop a significant immunity.

Fazekas de St. Groth and Donnelley (1950) have demonstrated that, in mice, there is no development of antibodies to the soluble CF antigen of influenza virus (the S30 component of Weiner, Henle and Henle, 1946) except after experimental infection. The failure of the mice in this experiment to develop such CF antibodies, together with the relationship shown in Table 2 between the dose of aWS− given and the level of immunity subsequently developed, provide further evidence of the inability of aWS− to multiply in mouse lung tissue to any significant extent.

Immunization of mice with inactive aWS–.

Investigations were also made of the immunizing capacity of aWS– whose chick embryo infectivity had been destroyed or substantially reduced by various chemical and physical means.

aWS– allantoic fluid was subjected to the following treatments: (a) Heated at 56° C. for 30 minutes. (b) Exposed to a Hanovia Fluorescent Model II Ultra-Violet (UV) lamp for 3 minutes at a distance of 17 cm. from the bulb. (c) Treated with formaldehyde (1/2000 dilution of HCHO) at 37° C. for two hours.

The HA titre of the unheated allantoic fluid was 450 and its EID_{50} approximately $10^{8.0}$. After heating at 56° C. the HA titre fell to 200 and the EID_{50} to $10^{0.3}$. After treatment with UV and formaldehyde no fall in HA titre was detected. The EID_{50} of the UV treated preparation was $10^{0.3}$ and no live virus could be detected at all in the preparation treated with formaldehyde.

Groups of 8, 4-week-old mice were inoculated intranasally with 0·05 ml. amounts of 1 AD and 10 AD dilutions of the untreated virus and of each of the treated virus preparations. Fifteen days later each group of 8 mice was divided into 2 groups of 4; one of these groups was challenged by intranasal inoculation of 0·1 AD WS–, the other with 1 AD WS–. The lung lesion scores were assessed in the usual way and the results, which are shown in Table 4, indicate that the heated and the formaldehyde-treated virus completely failed to immunize the mice to challenge with WS–. The UV treated virus had a small but significant immunizing effect which was, however, much inferior to that shown by active virus.

TABLE 4.

Immunizing efficiency of inactivated aWS–

Virus preparation used for immunization	Dose	Average MLP scores following challenge with WS–		
		Challenge dose (AD)		
		0·01	0·1	1·0
Active aWS–	1 AD		2·1+	0·5
	10 AD		0·0	0·8
UV treated aWS–	1 AD		2·3	4·6
	10 AD		1·1	1·2
Heated aWS–	1 AD		4·3	4·5
	10 AD		4·7	5·0
HCHO treated aWS–	1 AD		4·2	4·8
	10 AD		3·2	5·0
nil		2·7	4·4	

+ One non-immune mouse in this group with a score of 4·0 considerably raised the group average.

Infection of mice with sub-lethal doses of WS–.

Intranasal inoculation of mice with aWS– usually results in immunity to subsequent infection with WS– and in a rise in serum anti-HA levels, but fails to cause a rise in antibody titres to the soluble CF antigen. The question of whether infection of mice with sub-lethal doses of WS– would cause an increase in the levels of CF antibody was investigated in the following experiment.

Groups of 12 mice were inoculated intranasally with 0·05 ml. amounts of aWS– allantoic fluid diluted to 1 AD $\times 10^{-6}$, 1 AD $\times 10^{-5}$ and 1 AD $\times 10^{-4}$. Seven days later 6 mice in each group were killed to assess the MLP scores. The remaining 6 were kept for 15 days after inoculation and were then killed by bleeding out from the axillary vessels under ether anaesthesia. Their lungs were examined for lesions and the sera titrated for CF antibody, with results shown in Table 5. It is clear that 50 p.c. of the mice which received the largest sub-lethal dose of WS– in this experiment developed low titres of CF antibody.

TABLE 5.

Production of CF antibody by mice inoculated with sublethal doses of WS–.

Dose of WS–	Average MLP scores		CF antibody titres 15 days after inoculation
	Time after inoculation		
	7 days	15 days	
1 AD $\times 10^{-6}$	0·0	0·0	all <5
1 AD $\times 10^{-5}$	0·6	0·0	all <5
1 AD $\times 10^{-4}$	1·6	0·9	<5, <5, 4+, 15, <5, 7·5

+ Trace reaction at 1/5 dilution.

Failure of aWS– to produce toxic lesions in mouse lung.

The ability of influenza virus, when inoculated intranasally in large amounts into mice, to give rise to "toxic" consolidation unassociated with virus proliferation was first described by Sugg (1949). aWS– virus, diluted to 100 AD, 10 AD and 1 AD, was inoculated into groups of mice which were killed 2 days later. In none of these groups was there any evidence of toxic congestive lung lesions.

Immunization of ferrets with aWS–.

Following these experiments on the immunization of mice, two ferrets were bled (serum 1 in Table 6) and inoculated intranasally with undiluted aWS– allantoic fluid. Two days later the ferrets were anaesthetized and the anterior nares washed out with approximately 10 ml. of nutrient broth. The washings together with antibiotics were inoculated allantoically into chick embryos. In neither case could virus be detected following inoculation of a 10^{-1} dilution.

Fourteen days after inoculation of aWS– the animals were bled again (serum 2) and inoculated intranasally with undiluted WS– allantoic fluid. The temperatures were taken twice daily for the next three days and showed no deviation from the normal range. Nasal washings taken in one case 2, in the other 3, days after WS– inoculation again failed to yield virus on inoculation into chick embryos at 10^{0} and 10^{-1} dilutions. The ferrets were bled out 14 days after the WS– inoculation (serum 3) and all serum samples tested for their anti-HA titres. In addition, the serum from ferret 2 was tested for CF antibody and for neutralizing antibody for WS–. The results are shown in Table 6. Both animals showed an increase in the anti-HA titres of their sera following both the primary and secondary inoculation. In the case of ferret 2 this antibody rise was confirmed by *in ovo* determinations of the virus neutralizing activity of the various sera. It is interesting to note that inoculation of aWS– was not followed by a rise in CF antibody titre but that the second inoculation of WS– was followed by a rise in titre from <5 to 15. This would suggest that some multiplication of WS– took place in the ferret despite the presence of neutralizing antibody in the serum. It is possible, however, that subsequent neutralization of the virus by antibody prevented its isolation from the nasal washings taken 2 days after infection.

TABLE 6.

Serological response of ferrets inoculated intranasally with aWS– followed 14 days later by WS—.

Serum No.	Anti-HA		CF antibody Ferret 2	EID_{50} WS– Virus neutralized (log 10) Ferret 2
	Ferret 1	Ferret 2		
1	40	<10	<5	0·1
2	320	240	<5	3·0
3	480	450	15	6·1

Recombination between aWS– and WS–.

In the previous paper (Lind and Burnet, 1958) it was briefly reported that recombination would take place between WS– and aWS– giving rise to strains of intermediate levels of mouse virulence. A more extensive study of the phenomenon has given results which confirm those reported earlier.

The experiments were carried out in de-embryonated eggs (DEE) using the two virus strains aWS– and a fully mouse virulent revertant strain of WS– (obtained by limit dilution titration of low dilution passage strain 6A, Lind and Burnet, 1958). Each virus was diluted to the 15 AD level and the dilutions mixed in the proportions aWS– : WS– :: 5 : 1 before inoculation into DEE. Other DEE were inoculated with each of the two virus strains alone. The DEE were washed out 1 hour later, refilled with bathing fluid and harvested 6 hours after infection.

A pool of the harvests from the DEE inoculated with aWS– alone failed to produce lesions when inoculated intranasally into mice. The harvest from the DEE which had received only WS– killed all mice inoculated intranasally with 1 AD dilution with an average MLP score of 4·5.

The harvest from the doubly infected DEE was titrated to limit dilution and the 8 limit dilution (LD) fluids obtained were inoculated intranasally into mice at the 1 AD dilution level. Six of these gave MLP scores of either 0 or > 4 characteristic of the two parent strains. The other two gave intermediate scores of 3·6 and 2·4. The fluid giving the score of 3·6 was not investigated further, but that giving the score of 2·4 (fluid 85762) was titrated intranasally in mice. As control, another fluid (85758), which gave an MLP score of 4·8, was titrated similarly. The details of the mouse lung titrations of these two fluids and of the LD fluids derived from them by a further limit dilution passage in the allantoic cavity are compared in Table 7. Fluid 85762 retained its character of intermediate mouse lung pathogenicity following one limit dilution passage.

TABLE 7.

Titration of fluids 85758 *and* 85762 *and the LD fluids derived from them by intranasal inoculation of mice.*

Dilution inoculated into mice	MLP scores			
	Inoculum			
	85758 (control)	1st LD 86206	85762	1st LD 86072
1 AD × 10^{-4}	1·2	0·2	0·0	0·0
1 AD × 10^{-3}	2·3	1·4	0·1	0·3
1 AD × 10^{-2}	2·8	2·3	0·9	0·5
1 AD × 10^{-1}	4·4	4·0	1·4	0·9
1 AD × 10^{0}	4·8		3·0	1·9

The ability of such an intermediate to revert to the full virulence of WS– on low dilution passage in the allantoic cavity was shown by the finding that an allantoic fluid obtained by inoculation of a 10^{-2} dilution of fluid 85762, when inoculated into mice at a dilution of 1 AD, killed all mice in the group with an MLP score of 4·5.

A second recombination experiment was carried out along similar lines. Three virus mixtures containing aWS– : WS– in the proportions 10 : 1, 5 : 1 and 2 : 1 were used to infect the DEE and the harvests taken 7 hours after infection were titrated to limit dilution in the allantoic cavity. The resulting LD fluids were inoculated intranasally into mice at two different dose levels, 1 AD × 10^{-3} and the conventional level of 1 AD.

In the test at the 1 AD dose level only two fluids gave MLP scores between 0 and > 4. One of these, fluid 91953, obtained from DEE inoculated with the 10 : 1 virus mixture gave an MLP score of 0·6; the other, fluid 92000, obtained from DEE inoculated with the 2 : 1 mixture gave a score of 2·6. The harvest from the DEE inoculated with 5 : 1 mixture yielded only LD fluids of WS– type, i.e. which gave MLP scores > 4. Inoculation of all LD fluids into mice at the 1 AD × 10^{-3} dilution level revealed differences which were obscured when the larger dose was used; several fluids produced only minimal lung lesions when inoculated at this dilution, although the majority gave average MLP scores close to that of 2·8 given by the WS– control. The details are shown in Table 8.

TABLE 8.

MLP scores of recombinant strains of intermediate mouse virulence.

Original fluid	1st LD	2nd LD	MLP scores	
			Dose	
			1 AD × 10^{-3}	1 AD
91953			0·05	0·6
	92155+			0·8
	92159			1·0
		92464		2·9
		92467		0·8
91956			0·5	4·5
91962			1·4	4·6
92000			0·1	2·6
	92174			0·9
	97534+			0·1
		97544		0·2
		97548		0·1
	97535+			1·2
		97629		0·1
		97631		0·1
92002			0·7	4·8
WS–			2·8	5·0
aWS–			0·0	0·0

+ Fluid used as inoculum for next LD passage.

Only those fluids which showed differences at the 1 AD dose level were studied further. These were both taken through two further limit dilution passages and one or more LD fluids tested for their mouse lung pathogenicity at each passage level. The results are included in Table 8. The MLP scores of fluid 91953 and its limit dilution passage derivatives indicated that this strain was of a fairly stable intermediate level of mouse pathogenicity. The limit dilution passage derivatives of fluid 92000 showed a marked fall in MLP scores

when compared with the parental virus. This is in agreement with previous findings that virus strains which acquire virulence by recombination frequently show a decrease in their virulence characteristics on passage (Burnet and Lind, 1954b).

Alternate mouse lung/limit dilution passage of fluids 91953 and 92000 resulted in a swift emergence of virus with full mouse virulence. This is in contrast to the finding that revertant WS– strains of intermediate virulence obtained by serial low dilution passage of aWS– tended to retain their virulence characteristics when passaged under these conditions (Lind and Burnet, 1958). However, in Lind and Burnet's passage series mice were sacrificed 2 days after inoculation to provide material for limit dilution allantoic passage, whereas, in the present series, the mouse lungs were not removed until the time of final scoring, i.e. 7 days after inoculation. This would give an opportunity for any virus of high mouse virulence which formed only a minority of the total virus population in the inoculum to grow at the expense of less virulent types and so dominate the final yield from the mouse lungs.

DISCUSSION.

The strain aWS– was obtained as a mutant of the mouse virulent influenza strain WS– from which it differs only in its lack of mouse lung pathogenicity (Lind and Burnet, 1958). In Lind and Burnet's paper the conclusion was reached that the mouse virulence of the WS– strain depended on a number of genetic factors and that mutation to aWS– did not involve the loss of any of these factors, but resulted from the introduction of a suppressor gene controlling their activity into the genetic mechanism of the virus. aWS– was found to revert to the mouse virulent WS– form and this could most clearly be shown by serial low dilution passage of aWS– in the allantoic cavity of the chick embryo which resulted in the gradual dominance of the yield by WS– strains of various levels of mouse virulence. It was concluded that the level of mouse virulence reached by the revertant strains depended on the number of genetic factors concerned with mouse virulence which were released from the control of the suppressor gene.

In this paper the nature of the loss of virulence in aWS– has been studied in regard to the action of the virus on mouse lung. It seemed that there were two ways, not mutually exclusive, in which this loss of virulence could have occurred; either the virus could have retained its ability to multiply in mouse lung but have lost the power to produce any recognisable pathogenic change, or it could have lost its ability to multiply in these particular host cells. The ability of influenza virus to multiply in mouse lung without producing lesions has been observed in studies of adaptation of the virus to growth in mouse lung (Hirst, 1947; Sugg, 1949; Briody *et al.*, 1955).

The experimental results presented here make it clear that the failure of aWS– to produce lung lesions is due to a loss of ability to multiply in mouse lung tissue. Support for this conclusion comes from several experimental findings. Firstly, it was not possible to reisolate the virus from the lungs of mice 1, 2 and 3 days after intranasal inoculation except occasionally in very small amounts which could have represented residual inoculum or possibly the result of multiplication of small amounts of revertant virus. Secondly, it was found that while intranasal inoculation of mice with aWS– protected all but a few against the effects of a subsequent challenge with WS–, this protection was proportional to the dose of aWS– administered, and that while neutralizing antibodies to WS– appeared in the sera there was no evidence of the development of complement fixing antibody to the soluble antigen of influenza virus type A. It has been shown by Fazekas de St. Groth and Donnelley (1950) that antibodies to the soluble complement fixing antigen only appear when virus has multiplied in the mouse. Low titres of complement fixing antibody were produced in the sera of some mice following inoculation of sublethal doses of WS–.

Although aWS– failed to multiply in mouse lung it was adsorbed to mouse lung cells at the same rate as the virulent WS–.

Intranasal inoculation of large amounts of aWS– was not followed by the development of the "toxic" congestive lesions commonly noted when large amounts of influenza virus are instilled intranasally into mice.

Investigation of the serological response of ferrets to the intranasal inoculation of aWS– gave results similar to those obtained with mice. The sera showed an increase in antibody to WS– as determined by both anti-HA and *in ovo* techniques. A second intranasal inoculation of WS– was followed by a further antibody rise. Sera from one ferret were tested for their titres of antibody to the soluble influenza type A complement fixing antigen. No rise in complement fixing antibody was detected following aWS– inoculation, but the subsequent inoculation of WS– was followed by a significant increase in titre.

Several experiments on recombination between fully revertant WS– and aWS– were included in this study. Strains of various levels of mouse lung pathogenicity between those of the fully virulent WS– and the non-virulent aWS– parent strains were obtained which were classified as recombinants. It would, therefore, seem possible that, in the course of low dilution passage of aWS– in the allantoic cavity, strains of intermediate levels of mouse virulence could arise by recombination between aWS– and revertant WS– strains. However, the very small number of recombinants obtained in these experiments suggests that recombination plays little or no part in the reversion of aWS– to WS– under these conditions.

In agreement with the results of Lind and Burnet (1958), there was no evidence in these experiments of any interference by aWS– with the growth of smaller doses of WS– inoculated simultaneously into allantoic cells.

Acknowledgments. The author is indebted to Professor Sir Macfarlane Burnet, O.M., F.R.S., for his advice and criticism throughout this work, to Miss Beverley Perry, who performed the complement fixation tests, and to Miss Judith Gorrie for technical assistance.

REFERENCES.

Ada, G. L., Donnelley, M., and Pye, J. (1952): Austral. J. exp. Biol., 30, p. 301.
Briody, B. A., Cassel, W. A., and Medill, M. A. (1955): J. Immunol., 74, p. 41.
Burnet, F. M., and Lind, P. E. (1954a): Austral. J. exp. Biol., 32, p. 153.
Burnet, F. M., and Lind, P. E. (1954b): Nature, 173, p. 627.
Donnelley, M. (1951): Austral. J. exp. Biol., 29, p. 137.
Fazekas de St. Groth, S. (1948): *Ibid.*, 26, p. 29.
Fazekas de St. Groth, S., and Donnelley, M. (1950): *Ibid.*, 28, p. 45.
Hirst, G. K. (1947): J. exp. Med., 86, p. 357.
Lind, P. E., and Burnet, F. M. (1957): Austral. J. exp. Biol., 35, p. 57.
Lind, P. E., and Burnet, F. M. (1958): *Ibid.*, 36, p. 159.
Reed, L. J., and Muench, H. (1938): Amer. J. Hyg., 27, p. 493.
Stone, J. D. (1949): Austral. J. exp. Biol., 27, p. 337.
Sugg, J. Y. (1949): J. Bact., 57, p. 399.
Weiner, M., Henle, W., and Henle, G. (1946): J. exp. Med., 83, p. 259.

THE DETERMINATION OF UREA IN BLOOD

by HENRY FRANCIS HOLDEN[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication 15th November, 1958.*)

SUMMARY.

A colorimetric determination of blood urea, suitable for diagnostic laboratories uses diacetyl monoxime but less acid than other allied methods and no oxidant. The colour is stable. The method has been compared with the urease method to which some modifications are suggested.

INTRODUCTION.

The diagnostic determination of blood urea is carried out in many laboratories with the action of the urease in soya-bean meal and subsequent aspiration of the evolved ammonia into standard acid or with a purified urease on a protein-free blood filtrate and nesslerization of the liberated ammonia in the same vessel. The sundry disadvantages of these procedures have led to numerous modifications and to the appearance of several other methods. These include the reaction of urea with phenol and NaOCl to produce a blue colour (Van Slyke and Hiller, 1933), methods based on the precipitation of urea with xanthydrol and a group of methods based on the reaction of urea with diacetyl monoxime ("DAMO") described by Fearon (1939) or with other allied compounds. These DAMO reactions in general need much concentrated mineral acid and an oxidizing agent to develop the colour. It appears from the critical paper by Rosenthal (1955) that in such methods the concentration of the various oxidants and the time of reaction must be so nicely adjusted and the coloured products are so rapidly destroyed by the same oxidants that the procedures are awkward for harassed analysts. Papers by Friedman (1953), Wheatley (1948), Koritz and Cohen (1954), Iuchi and Miyajo (1955), Ormsby (1942) and Barker (1944) refer to one or both of these aspects of the subject.

Archibald (1945) used iso-nitroso propio-phenone and a mixture of sulphuric and phosphoric acids to produce a stable red colour with urea without the use of any added oxidant. The reagent is not available in many places and a large amount of strong acid is needed. This paper, which is written in the

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

interests of clinical analysis, offers a method in which a coloured derivative, as stable as those described by Wheatley or Archibald is produced with DAMO and little acid, phosphoric acid alone being used; no oxidant is required and though the use of N-phenyl anthranilic acid as an intensifier was tried, it was not found to be necessary for clinical analysis. Where only very small blood specimens are available, Wheatley's method appears to be the best. The investigation also brought to light some useful modifications to the urease method for those who prefer titration to colorimetry.

Materials and Methods.

The only special apparatus that is desirable is a number of "Pyrex" test-tubes 125 mm. long and 14-15 mm. bore. A portion of each tube is heated and drawn out till the bore is about 8 mm.; a mark is made with a diamond pencil in the narrow portion such that the crude graduated flask so made contains a volume of fluid suitable for the colorimeter available. The one used for this work is an old "home-made" photoelectric colorimeter with a set of glass filters and a cuvette (a selected test-tube) requiring 5 ml. of solution. The tubes were therefore graduated to contain 6·0 ml. of liquid. Under these conditions, using the blue filter and 0·5 ml. of blood filtrate for a determination, a half-scale deflection corresponds to a blood containing 63 mg. of urea per 100 ml. It has been found to be unnecessary to stopper the tubes during the reaction. The blood filtrates were made with Somogyi's (1930) "alternative" method (see also Barker, 1944). The DAMO was prepared in the laboratory by the action of ethyl nitrite on ethyl methyl ketone; after removal of other unwanted matter at 50° C. with a water pump the DAMO was sublimed at about 0·1 mm. It seems probable that the stable colour was favoured by the circumstances that the protein-precipitating reagent was free from any trace of reducing substances and that the DAMO was purified by a simple but very effective method.

The reagents are as follows:

1. 1 gm. of DAMO in 100 ml. water.
2. Ortho-phosphoric acid. 141·5 gm. of "ortho-phosphoric acid 100 per cent. A.R." mixed with 20 ml. of water.
3. A mixture of 1 vol. of 1 with 4 vols. of 2; this is mixed shortly before the determinations are to be made and should not be kept overnight.

For an exact determination on an unknown blood take two of the tubes. In the first, put 0·5 ml. and in the second 0·2 ml. of the blood filtrate. In the second put also 0·3 ml. of water. To each add 1·0 ml. of reagent 3. Mix carefully. Place the tubes in a boiling water-bath for 60 minutes. Remove the tubes and cool to room temperature. Add water to the mark on each tube, mixing carefully. Transfer the contents in turn to the cuvette of the photometer and read. The use of two tubes ensures that the range up to 250 mg. of urea per 100 ml. of blood is adequately covered, but for most diagnostic work the first tube can probably be omitted. It is needed chiefly for values less than 30 mg. per 100 ml. Where the urea concentration is approximately known other arrangements may be substituted. The proportions of DAMO, acid and water have been found by experiment to be those most suitable. A calibration curve is valid only for one set of proportions.

To test the suitability for diagnostic purposes of this DAMO method it was decided to compare its results with those of a urease-titration method on a number of specimens of blood, some with urea added to them. The experience thus gained has suggested some variations in the latter method from the manner in which it is sometimes now carried out. These variations are to be found in the main in the neglected literature on the subject. Very precise equipment, such as that of Neri (1930) or of Kay and Sheehan (1934), is too fragile for general use; only those procedures suitable for diagnostic work were studied. To economize in blood and reagents the method was scaled down. The reaction and absorption tubes were 15 cm. long by 23 mm. bore. Since traces of spray were noticed (Sumner, 1916; Kay and Sheehan, 1934) the traps were altered as shown. To diminish the loss of ammonia through incomplete absorption the aspiration lasted one hour at a diminished rate (Fiske, 1915). Since some samples of rubber tube appeared to be somewhat permeable to ammonia a slow current of air was drawn through the apparatus throughout the incubation period (Van Slyke and Cullen, 1914). The acid and the reaction tubes and their respective bubblers were marked and kept entirely separate during cleaning (Van Slyke and Cullen, 1916). The absorption equipment was simply rinsed thoroughly with distilled water and dried after each experiment. A compact metal rack (Neri, 1930) was made to hold the reaction and the absorption tubes in two parallel lines 10 cm. apart. The centres of the tubes in each line were 37 mm. apart and situated opposite the spaces between the tubes in the opposite line. A gap in the frame of the rack (Neri, 1930) allows it to sit on the edge of a rectangular commercial water-bath, with the bottoms of the reaction vessels immersed, while the acid vessels remain outside the bath at room temperature. A gas-washing bottle to remove ammonia from the entrant air is on the "acid" side of the rack. The saving in bench space would justify the slight expense of this arrangement.

For each determination one ml. of blood was measured with an Ostwald pipette and 3 ml. of 0·6 p.c. KH_2PO_4 solution, 6 drops of octyl alcohol and 0·3 gm. of finely ground soya-bean meal added. After 30 minutes incubation at 45° C. the ammonia was liberated with 7 ml. saturated K_2CO_3 solution and aspirated into 10 ml. of 0·1 N. H_2SO_4.

Further studies showed that for this batch of soya-bean 30 minutes' incubation at 37° C. with 0·1 gm. of meal is ample, thus avoiding the need for a special water-bath at 45° C. Beans, however, appear to vary greatly in quality by the time they arrive in the laboratory.

For the comparison of the two methods each was done in duplicate. An additional pair of tubes was put in the urease series to correct for traces of ammonia in the blood and reagents and (possibly) traces of spray. Since the two methods were calibrated independently, the one against standard H_2SO_4 and the other against pure urea the results indicate that whatever be the nature

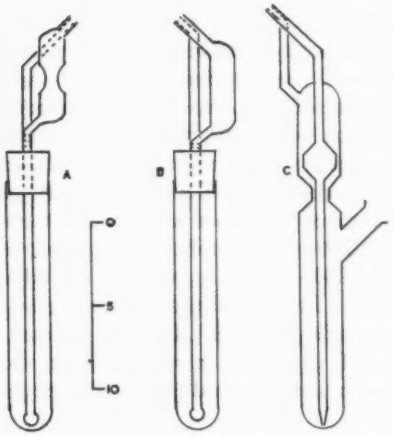
of the small remaining errors they do not invalidate this DAMO method for diagnostic purposes (Table 1).

TABLE 1.

Urea in mg. per 100 ml. of blood.

Urease ...	34	38	49	51	93	101	147	185	215	249
DAMO ...	35	36	48	54	100	98	145	184	201	285

Further studies on the urease method confirmed the view that the reagents might be diminished to 2 ml. of KH_2PO_4 solution and 4 ml. of saturated K_2CO_3 solution without affecting the results. To overcome the difficulty of placing the opening of the bubbler as near as possible to the bottom of the large tube without damage to either, a new type of reaction vessel was made and tested, while retaining the form of the absorption vessel shown at B. As can be seen at C in the sketch the bubbler is reduced to a robust tube drawn out to a tip. This tube is sealed through the top of the reaction vessel. A tubulure, closed with a rubber stopper serves for the admission of the solutions and the powdered soya-bean. The effect of a spray trap is achieved by constricting the vessel above the tubulure and blowing a bulb on the inner tube just above the constriction. When tested with reaction vessel A, it was found that both gave the same analytical results and when grossly over-driven passed much less spray than the usual straight-path spray trap under the same conditions. It appears easier to add the K_2CO_3 solution without accidental loss of NH_3; after use it is cleaned by drawing a stream of water through it with a water vacuum pump.



A. Reaction vessel.
B. Absorption vessel.
C. Reaction vessel with built-in tube.

Acknowledgments. I wish to thank Miss B. Splatt and Dr. S. Weiden for their kindness in providing me with specimens of blood.

Drs. Iuchi and Miyajo kindly sent to this Institute an English translation of their paper.

REFERENCES.

Archibald, R. M. (1945): J. biol. Chem., 157, p. 507.
Barker, S. B. (1944): *Ibid.*, 152, p. 453.
Fearon, W. R. (1939): Biochem. J., 33, p. 902.

Fiske, C. H. (1915): J. biol. Chem., 23, p. 455.
Friedman, H. S. (1953): Anal. Chem., 25, p. 662.
Iuchi, I., and Miyajo, T. (1955): Igaku to Seibutsugaku, 36, p. 121.
Kay, W. W., and Sheehan, H. L. (1934): Biochem. J., 28, p. 1784.
Koritz, S. F., and Cohen, P. P. (1954): J. biol. Chem., 209, p. 145.
Neri, A. (1930): Biochem. terap. sper., 17, p. 379.
Ormsby, A. A. (1942): J. biol. Chem., 146, p. 595.
Rosenthal, H. L. (1955): Anal. Chem., 27, p. 1980.
Somogyi, M. (1930): J. biol. Chem., 86, p. 655.
Sumner, J. B. (1916): *Ibid.*, 27, p. 95.
Van Slyke, D. D., and Cullen, G. E. (1914): *Ibid.*, 19, p. 211.
Van Slyke, D. D., and Cullen, G. E. (1916): *Ibid.*, 24, p. 117.
Van Slyke, D. D., and Hiller, A. (1933): *Ibid.*, 102, p. 499.
Wheatley, V. R. (1948): Biochem. J., 43, p. 420.

PRESENCE OF K-VIRUS IN WILD MICE IN AUSTRALIA

by DOLORES HOLT

(From the Queensland Institute of Medical Research, Brisbane).

(*Accepted for publication 3rd December, 1958.*)

SUMMARY.

A virus, which causes fatal pneumonitis in suckling mice, was isolated from three of nine *Mus musculus* trapped in a food-processing factory. Twenty-four *Mus musculus* from other sources around Brisbane were examined, but there were no isolations of the virus.

It is indistinguishable immunologically and histopathologically from K-virus.

INTRODUCTION.

K-virus, which causes fatal pneumonitis in suckling mice, was isolated in Maryland, U.S.A., five times in a total of 22 attempts from female C_3H mice carrying the Bittner milk agent (Kilham and Murphy, 1953). Altogether, there were 60 unsuccessful attempts to isolate the virus from male and female C_3H, C-strain and hybrid mice, some known to carry the Bittner milk agent and others to be free of it. There is no evidence to prove or disprove a relationship between K-virus and the Bittner milk agent.

A virus, which is immunologically indistinguishable from K-virus, was isolated from three of 33 *Mus musculus* investigated in a search for infective agents in Brisbane. It is believed that it has not been recorded in Australia before.

Materials and Methods.

Isolation of the virus. The 33 mice were trapped alive in various warehouses, factories and private homes around Brisbane, and were killed by town gas. A saline suspension of liver, spleen, kidney, heart, lung and brain from each mouse was inoculated intracerebrally into four adult mice, and intraperitoneally into four adult mice and two litters of suckling mice. The virus was isolated in suckling mice from three of nine mice caught in one food-processing factory. Details of these mice are shown in Table 1.

Experimental mice. The strain of mice used has been bred at the Queensland Institute of Medical Research for nine years without the introduction of an outside strain (Derrick *et al.*, 1954). The suckling mice were generally less than 48 hours old when used, and the average litter number was seven. In suckling mice, the intraperitoneal inoculum was 0·05 ml. and the intracerebral, intranasal and subcutaneous inocula 0·01 ml. In adult mice, the intraperitoneal inoculum was 0·5 ml. and the intracerebral and intranasal inocula 0·03 ml. A liver-spleen-lung emulsion was inoculated intracerebrally into suckling mice for routine passages.

Culture media. Blood agar, cooked meat medium, MacConkey agar, Sabouraud's agar, serum agar, nutrient broth, and Edward's medium for the cultivation of pleuropneumonia-like

TABLE 1.

Particulars of mice from the food-processing factory.

Mouse	Date captured	Sex	Weight	Virus isolation	Neutralization test
WM17	5/6/57	♂	14 g.	+	+*
WM18	5/6/57	♂	18 g.	+	+
WM19	7/6/57	♀ (pregnant)	16 g.	+	+
WM21	7/6/57	♂	17 g.	−	+
WM29	30/5/58	♂	10 g.	−	−
WM30	4/6/58	♂	10 g.	−	−
WM31	10/6/58	♂	7 g.	−	−
WM32	17/6/58	♂	13 g.	−	−
WM33	17/6/58	♂	19 g.	−	+

* + indicates neutralization of 100 LD_{50} of WM18 virus.

organisms were used in attempts to grow bacteria and fungi, and were incubated aerobically and anaerobically at room temperature and 37° C.

Preparation of suspensions for heat and germicidal tests. Heat resistance tests were carried out with 10 p.c. infective liver-spleen-lung suspensions cleared by centrifugation. The suspensions were then stored overnight at—70° C., cleared by spinning at 3,000 r.p.m. for 15 minutes, and ampouled. The ampoules were completely immersed in water at the desired temperature.

Germicidal resistance tests were carried out by the method described by Stanley *et al.* (1951).

Preparation of immune sera. Ten per cent. suspensions of liver, spleen and lung from infected mice were centrifuged at 2,500 r.p.m. for 15 minutes, and 2·0 ml. of the supernate injected intravenously into 1800 gm. rabbits. Three doses were given at weekly intervals, the rabbits being bled seven days after the last injection.

Neutralization test. The virus used in the test was diluted in 10 p.c. rabbit serum-saline, and equal volumes of virus dilutions and serum were allowed to stand at 37° C. for 30 minutes (Kilham and Murphy, 1953). The virus controls were allowed to stand for 30 minutes in normal rabbit serum at 37° C. The sera were inactivated at 60° C. for 30 minutes.

Cross-immunity tests. Adult female mice were divided into three groups, and inoculated subcutaneously (0·3 ml.) or intraperitoneally (0·5 ml.) with 10 p.c. liver-spleen-lung emulsions of WM18 virus, K-virus, and saline, respectively. They received eight injections in the course of three weeks, and thereafter one each month (Melnick *et al.*, 1950). They were kept in groups of four females per male, and observed daily for appearance of young. Within 48 hours of birth, the newborn mice were inoculated intracerebrally with 10^4 LD_{50} of virus.

EXPERIMENTAL.

Infection in suckling mice. Suckling mice inoculated intraperitoneally with pooled material from infected wild mice died in 12 to 15 days, a few hours after the development of laboured respiration. At autopsy, the lungs were a deep plum colour, all lobes being affected, and pleural fluid was present. All other organs appeared normal, and the stomachs were full of milk. Mice killed prior to the development of the laboured respiration had pale lungs, with petechiae.

From these mice, the virus was maintained by passaging liver, spleen and lung intraperitoneally and intracerebrally. The incubation period was reduced with routine passage, and, after eight passages, inoculated mice died in eight days on intraperitoneal inoculation. At autopsy, the findings were the same as after the primary passage.

When a 10 p.c. suspension of liver, spleen and lung was inoculated intracerebrally into suckling mice of different age groups, mortality was 48/48 in seven-day-old mice, 32/32 in eight-day-old mice, 25/32 in nine-day-old mice, 4/51 in ten-day-old mice, and 0/33 in mice eleven days old. There were no deaths after intraperitoneal inoculation of mice older than ten days.

Histology. The histopathological findings in suckling mice were essentially similar to those described by Fisher and Kilham (1953). They were almost entirely limited to the lungs, regardless of the route of inoculation. A striking change was seen in many endothelial cells in small arterioles and septal capillaries (Fig. 1). Their normally elongated nuclei were greatly swollen, and tended to become vesicular, with an outer ring of chromatin and frequently a central body (Fig. 2). The nuclear changes were more clearly demonstrable in Zenker- than in formalin-fixed tissue. In places the septa were thickened by infiltration of mononuclear cells. Alveolar spaces were empty, containing neither exudate nor inflammatory cells. There was no necrosis or fibrosis, and no perivascular or peribronchial massing of lymphocytes.

An occasional cell in the liver appeared to show "ballooning" of the nucleus, but spleen, kidney, heart and brain appeared normal.

Nothing abnormal was seen in sections from weaned mice inoculated with infective liver-spleen-lung emulsion.

Comparison of methods of inoculation. To compare methods of inoculation, a 10 p.c. suspension of liver, spleen and lung was inoculated intraperitoneally, intracerebrally and intranasally. The clinical and pathological features were identical; but the incubation period was shortest with the intracerebral route, and titration (Table 2) showed it to be most sensitive.

TABLE 2.

Dilution of WM18 virus preparation killing 50 p.c. of mice after inoculation by various routes.

Method of inoculation	Titre for LD_{50}	No. of mice tested at each dilution	Incubation period at 10^{-1} dilution. Days
Intracerebral	$10^{-8.0}$	16	6
Intraperitoneal	$10^{-6.0}$	16	8
Intranasal	$10^{-5.8}$	16	8

Concentration of the virus in tissues following intracerebral inoculation. To determine the concentration of virus in various tissues, six infected mice were bled out seven days after inoculation, and tenfold dilutions of pools of each

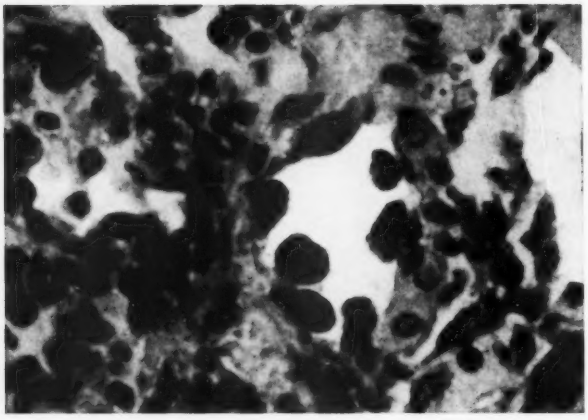


Fig. 1. Formalin-fixed section demonstrating large endothelial cells. H. & E., x 780.

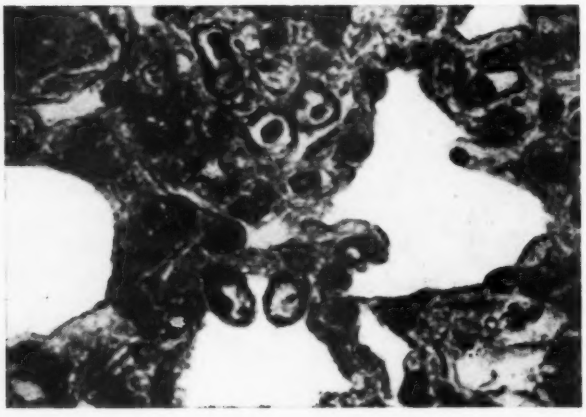


Fig. 2. Zenker-fixed section demonstrating intranuclear inclusions. H. & E., x 1,850.

tissue were inoculated intracerebrally into 12 suckling mice for each dilution. Mice were examined daily to determine the LD_{50}, those dying before four days being discarded. The results are shown in Table 3. The highest infectivity was

TABLE 3.

Concentration of virus in the tissues of suckling mice following intracerebral inoculation.

Tissue	Log. LD_{50}/gram	Incubation period at 10^{-1} dilution of tissue. Days
Blood	9·0	10
Brain	6·2	11
Liver	>9·0<11·0	10
Lung	11·0	7
Heart	5·0	10
Kidney	5·3	13
Spleen	8·4	10
Pleural fluid	>7·0<9·0	9
Urine	>3·0<5·0	14
Faeces	5·0	14

found in the lung, and this is supported by the shortness of the incubation period. Although brain to brain transfers were positive, there were no clinical or pathological signs of CNS involvement.

Re-isolation of the virus. To confirm that the wild mice were the source of the virus, stored material from each of WM17, 18 and 19 was re-inoculated into suckling laboratory mice – once with organ emulsion stored for three months at –20° C., and again with emulsion stored for 10 months at – 70° C. Re-inoculations were followed by the typical effects of K-virus.

Properties of WM18 virus. The agent was suspected to be a virus, when it was found to be resistant to the action of penicillin and streptomycin at concentrations of 5,000 units/ml., and when cultures for bacteria of material known to contain the infective agent were uniformly sterile. This was confirmed, when WM18 virus was shown to pass through Seitz E.K. filters. The incubation period for suckling mice inoculated with filtered material was the same as for those inoculated with unfiltered material.

WM18 virus is stable, and, like K-virus (Kilham, 1952), tolerates frequent freezing and thawing. A liver-spleen emulsion was still lethal for suckling mice

TABLE 4.

Heat sensitivity tests.

Temperature	37° C		60° C		70° C		98° C
Time of exposure	5½ days	7 days	10 hrs.	24 hrs.	3 hrs.	4½ hrs.	2 mins.
Results	8/8	8/8	28/28	16/16	11/14	0/16	0/8

Numerator = number of suckling mice dead.
Denominator = number of suckling mice inoculated.

after standing at room temperature for 11 weeks. The virus is highly resistant to heat, surviving, for example, for 24 hours at 60° C. Details of temperature and times of exposure are set out in Table 4.

Germicidal resistance tests revealed that the virus is resistant to 70 p.c. ethanol, 0·25 p.c. phenol, and 0·5 p.c. formalin, but is inactivated by 2 p.c. lysol (Table 5). Like K-virus (Kilham and Murphy, 1953), WM18 virus resists exposure to 20 p.c. ether for 30 minutes.

TABLE 5.
Germicidal sensitivity tests.

Germicide	Time of contact	
	1 hour	24 hours
70 p.c. ethanol	8/8	8/8
2 p.c. lysol	0/8	0/8
0·5 p.c. formalin	8/8	8/8
0·25 p.c. phenol	8/8	8/8

Numerator=number of suckling mice dead.
Denominator=number of suckling mice inoculated.

Host range. Guinea-pigs inoculated intraperitoneally, intracerebrally and intranasally, and suckling albino *Rattus norvegicus* inoculated intraperitoneally and intracerebrally, showed no evidence of infection.

Ten- to 12-day-old chick embryos were inoculated on the chorio-allantoic membrane and in the amniotic sac, and six-day chick embryos in the yolk sac, with infective liver-spleen-lung material. Although virus was detectable by inoculating suckling mice with first passage material, the chick embryos did not die, and virus could not be detected in succeeding passages.

Weaned mice showed no signs of infection, whether inoculated intranasally, intracerebrally, intraperitoneally, or subcutaneously. Virus was detected up to 12 days after intraperitoneal inoculation, but not to 15 days. It could not be detected in second passage material.

That adult mice may become carriers of the virus, at least temporarily, is shown by the present isolations, as well as by those of Kilham, and by the excretion of virus in urine-faeces for at least four weeks by six survivors after laboratory infection.

Identification. Neutralization and cross-immunity tests were carried out with WM17, 18 and 19 viruses and K-virus to find their immunological relationships. The K-virus was obtained through the courtesy of Dr. L. Kilham, Division of Biologics Standards, Public Health Service, Bethesda, as a 10 p.c. suspension of liver and spleen, which had been lyophilized on 15/4/54, and was still viable when tested on 19/5/58.

Rabbit antisera prepared against WM17, 18 and 19 viruses and K-virus neutralized significant amounts of each virus (Table 6).

TABLE 6.

Cross neutralization between WM17, 18, and 19 viruses and K-virus.

Virus	WM17	WM18	WM19	K
Serum:	**>3·5**	>2·8	>3·2	>3
WM17	>3·5	**4**	>3·2	>3
WM18	>3·5	>2·8	**>3·2**	>3
WM19	>3·5	>4	>3·2	**>3**
K				

The results are expressed as log virus neutralized.

In the cross-immunity tests, there were no deaths in the infant mice born of virus-vaccinated mothers; but those born of saline-vaccinated mothers and inoculated with WM17, 18 and 19 viruses and K-virus died in six to eight days (Table 7). Although immunity indices were not obtained, the high order of resistance shown by suckling mice born of vaccinated mothers is in accord with the high indices obtained in the neutralization tests.

TABLE 7.

Cross-immunity tests.

Mother vaccinated with	WM18	K	Saline
Suckling mice inoculated with: WM17	0/16	0/16	12/12
WM18	0/20	0/24	16/16
WM19	0/16	0/12	12/12
K	0/16	0/18	15/15

Numerator = number of suckling mice dead.
Denominator = number of suckling mice inoculated.

Rabbit antisera prepared for use in the neutralization tests gave non-specific results, when attempts were made to utilise the complement fixation test.

Serological survey. No neutralizing antibodies were detected in the sera of 70 stock mice and eight wild *Rattus rattus*. Sera from 30 wild *Mus musculus* were tested, of which five were positive, all from mice trapped in the food-processing factory, including the three from which virus was isolated.

DISCUSSION.

The evidence indicates that the viruses isolated were of wild mouse and not stock mouse origin.

(1) The virus was repeatedly demonstrated in the original inocula.

(2) Neutralizing antibodies to WM18 virus were present in the sera of the mice from which the virus was isolated.

(3) No virus resembling WM18 has ever been demonstrated in the stock mice, although many inoculations from them into suckling mice have been made in the course of various investigations over nine years.

(4) Pools of lungs from eight healthy stock mice, and from 12 stock mice suffering from chronic lung disease, were inoculated into suckling mice, and three passages made. No deaths followed, and no macroscopic or microscopic signs of lung infection developed during the 21-day period of observation. The chronic lung disease to which older stock mice are liable resembles that caused by the grey-lung virus of Andrewes and Glover (1945).

(5) No neutralizing antibodies to WM18 virus were found in 70 stock mice tested.

WM18 virus strongly resembles the K-virus in its properties and pathological effects produced in newly born mice. The striking peculiarity of both is the failure to cause illness in mice older than 10 days. The two viruses could not be distinguished by neutralization or immunity tests.

Since Kilham's isolations were all from female mice, the present isolations from male mice show that they may also become infected.

As pointed out by Fisher and Kilham (1953), no other pneumotropic virus of murine origin is lethal for suckling mice only. The histology and properties of such pneumotropic viruses as P.V.M., Nigg's virus, meningopneumonitis virus, and the virus of De Burgh *et al.* (1945) are quite unlike those of WM18 virus. The hepato-encephalomyelitis virus of Stanley *et al.* (1953) is lethal for suckling but not adult mice, but its impact is on liver and brain. Giant-cell formation is seen in the liver of weaned and, occasionally, suckling mice infected with the mouse hepatitis virus (Gledhill *et al.*, 1955), but, unlike infection with WM18 virus, the cells are multinucleated, and there is also necrosis of the liver.

This virus disease is enzootic at the factory, neutralizing antibodies being present in a mouse captured there a year after WM18 was caught. The ability of the virus to survive outside the body, and its resistance to heat and germicides, would facilitate its transmission. It is excreted in urine or faeces for at least some weeks by mice that recover from experimental infection, and Kilham has shown that it is infectious for suckling mice by the nasal and oral routes. The mice probably become infected within a few days of birth. Those born of mothers that had not been infected probably die, and those with highly immune mothers may be completely protected; but mothers with a weak or waning immunity may confer only partial protection. Some of their progeny, and some of the unprotected infant mice, which happen to receive a minimal dose of virus or to become infected in the second week of life, would recover, and we may suspect that it is in these sections of the mouse population that the virus

is maintained in nature. More detailed studies of the duration of the urine-faeces carrier state after recovery would be illuminating.

This record extends the known occurrence of K-virus from the United States of America to Australia, suggesting that it may well be cosmopolitan in distribution, and it adds another virus lethal for infant mice to those already known in this country.

Acknowledgments. I wish to thank Dr. E. H. Derrick for the histological descriptions and for help in preparing the manuscript, and Mr. M. Smith for the histological preparations.

REFERENCES.

Andrewes, C. H., and Glover, R. E. (1945): Brit. J. exp. Path., 26, p. 379.
De Burgh, P., Jackson, A. V., and Williams, S. E. (1945): Austral. J. exp. Biol., 23, p. 107.
Derrick, E. H., Pope, J. H., Chong, S. K., Carley, J. G., and Lee, P. E. (1954): *Ibid.*, 32, p. 577.
Fisher, E. R., and Kilham, L. (1953): A.M.A. Arch. Path., 55, p. 14.
Gledhill, A. W., Dick, G. W. A., and Niven, J. S. F. (1955): J. Path. Bact., 69, p. 299.
Kilham, L. (1952): Science, 116, p. 391.
Kilham, L., and Murphy, H. W. (1953): Proc. Soc. exp. Biol. Med., N.Y., 82, p. 133.
Melnick, J. L., Clark, N. A., and Kraft, L. M. (1950): J. exp. Med., 92, p. 499.
Stanley, N. F., Dorman, D. C., and Ponsford, J. (1953): Austral. J. exp. Biol., 31, p. 147.
Stanley, N. F., Hayes, L., and Dorman, D. C. (1951): *Ibid.*, 29, p. 367.